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ABSTRACT

In eukaryotes, the genome is hierarchically packed inside the nucleus, which facilitates physical contact between cis-regulatory elements (CREs), such as enhancers and promoters. Accumulating evidence highlights the critical role of higher-order chromatin structure in precise regulation of spatiotemporal gene expression under diverse biological contexts including lineage commitment and cell activation by external stimulus.

Genomics and imaging-based technologies, such as Hi-C and DNA fluorescence in situ hybridization (FISH), have revealed the key principles of genome folding, while newly developed tools focus on improvement in resolution, throughput and modality at single-cell and population levels, and challenge the knowledge obtained through conventional approaches. In this review, we discuss recent advances in our understanding of principles of higher-order chromosome conformation and technologies to investigate 4D chromatin interactions.

INTRODUCTION

The genome is hierarchically organized at different genomic scales in the human cell nucleus to efficiently pack a two-meter-long polymer in the micrometer space (1-3). While folding the genome efficiently, it is critical to form functional domains for precise gene regulation at the right time and in the right cell type. A plethora of studies pinpoint chromatin folding as a major mechanism of gene regulation in normal development, and dysregulation of the chromatin conformation leads to diseases such as cancer (4).

'4D nucleome', the dynamics of three-dimensional architecture of genome across time and space (fourth dimension), has been an active area of the current research. To gain deeper insight into 4D nucleome regulation, the National Institute of Health (NIH) has launched the 4D Nucleome (4DN) Network in 2014 (5). The 4DN and other researchers have paved the way for new technologies to provide novel molecular and biophysical insights into spatial genome organization across time and space (5, 6). With the collective efforts, the principles of genome folding have been extensively studied via genomic approaches, such as chromosome conformation capture (3C) derived tools, namely Hi-C, and imaging methodologies based on DNA FISH. These technologies identified distinct functional chromatin domains at different genomic scales: chromosome territories, compartments, topologically associating domains (TADs) and chromatin loops (2, 4). Although the discovery of hierarchical domains sheds light on our understanding of chromosome folding in the nucleus and its functional aspects, unraveling the comprehensive mechanisms of higher-order chromatin architecture has been challenging due to the limited pool of available tools. Novel technologies besides 3C or FISH derivatives and improved conventional genomics and imaging-based approaches, with enhanced resolution, throughput and modality, have been developed. For example, ligation-free genomics

methods, such as genome architecture mapping (GAM), have been developed as new tools to overcome the bias of 3C-based approaches, which depend on proximity ligation of chromatin, capturing only simple chromatin interactions, but not the complex nature of the contacts (7, 8). In addition, imaging-based approaches which adapted Oligopaint FISH probes with super-resolution microscopy have provided high-resolution visualization of multiple chromatin interactions (9-12). The power of conventional tools has been enhanced recently to facilitate the examination of 4D genome with DNA methylation and transcription simultaneously (13-15). In contrast to microscopic approaches, which intrinsically provide single-cell information, 3C-based genomics technologies have been applied to cell populations. Cutting-edge efforts to develop single-cell genomics-based tools, such as single-cell Hi-C (scHi-C), uncovered the high variability of genome structure between individual cells, in contrast to prior studies that claimed stability of TADs across different cell types (16, 17). Technological revolutions continue to challenge and renew our understanding of concepts in genome architecture and function.

In this review, we discuss novel insights into higher-order chromatin organization, and technological advances to investigate 4D genome and their functional relevance in different biological phenomena.

HIGHER-ORDER GENOME STRUCTURE: PRINCIPLES AND PLAYERS

Chromosomes are folded at different scales of organization in the nucleus such as chromosome territories, compartments, TADs and chromatin loops (Fig.1). Higher-order chromatin folding is a non-random process, which is related to transcriptional activity (1-4, 18). In this section, we will illustrate the emerging concepts of genome folding and players in the hierarchical genome structure at different scales.

Chromosome territories and A/B compartments

During interphase, chromosomes occupy specific locations called chromosome territories inside the nucleus (Fig.1). Membrane-less organization of chromosome territories has been shown in a variety of different species and cell types via both microscopy and 3C-based technologies (19). Chromosome territories restrict inter-chromosomal interactions and promote intra-chromosomal interactions, even in regions separated by mega base-scale distances, although the territorial boundaries often intermingle (4). The territories consist of non-randomly positioned genomic regions known as compartments (Fig.1). Compartments were originally defined from one of the first chromosome conformation studies with 1MB resolution Hi-C as genome partitioning into two different compartments, A and B (20). The A and B compartments carry distinct epigenomic marks and transcriptional activity: The A compartment is characterized by the presence of active histone marks, open chromatin with actively transcribed genes, while the B compartment contains repressive histone marks, and closed chromatin with inactive genes. The A and B compartments are spatially segregated and associated with different nuclear structures. Microscopic studies show that the B compartment is mostly located at the nuclear periphery and surrounding the nucleoli, whereas the A compartment is located inside the nucleus, associated with nuclear bodies, such as speckles (Fig.1)(4). The preferential localization of B compartment to the nuclear periphery is mediated by lamin B receptor, lamin A and C, as the knockout of the three proteins induced re-localization of heterochromatin to the nuclear interior (21). While the absence of lamin proteins can change the location of B compartment, it does not result in global changes in gene expression related to B compartment or large-scale compartmental changes (22). Less is known about the mechanisms of preferential localization of the A compartment compared to the B compartment. The knockdown of Srrm2, a core scaffolding protein of nuclear speckle in

mouse hepatocytes disrupted intra-chromosomal interactions in the A compartment, suggesting an important role of nuclear speckles in the organization of chromosome compartments (23). Although the knowledge that the nuclear chromatin consists of distinct compartments with different epigenetic characteristics is now broadly accepted, the molecular mechanisms of chromatin compartmentalization remained an open question. A burst of recent studies suggests that phase separation may be one possible mechanism to explain the spatial segregation of A and B compartments (22). The compartments are membrane-less structures formed by chromatin fibers, which are long polymers composed of alternating A and B domains. Each domain can recruit different binding proteins including histones, RNA polymerase, and chromatin modifying factors, which leads to domain-domain compaction. Mechanisms such as polymer-polymer phase separation (PPPS) and liquid-liquid phase separation (LLPS) are implicated in the formation of compartments, especially LLPS which occurs when DNA or RNA binding proteins or nucleic acid itself, interact and condense into liquid-droplet like macromolecular structures (24). For example, heterochromatin protein (HP1) a/alpha isoform forms phase-separated droplets with liquid properties, exhibiting dynamics of LLPS *in vitro* and in *Drosophila melanogaster* embryos (25, 26). In *Schizosaccharomyces pombe*, Swi6, HP1 homolog, also induces liquid droplet formation in the presence of DNA molecules or nucleosomes (27). In addition, histone modifications such as H3K9me2 and H3K9me3 induce phase separation to facilitate chromatin compartmentalization by forming macromolecule-enriched liquid droplets with HP1, SUV39H1 and TRIM28 (28). Similarly, the polycomb complex, which establishes and maintains H3K27me3 marks, also induced phase separation by forming droplets *in vitro* (29). Reconstituted chromatin intrinsically undergoes phase separation and forms highly concentrated liquid-like droplets under physiological salt conditions, mediated by linker histone H1-promoting

6

chromatin compaction (22). These multiple lines of evidence indicate a critical role of LLPS in the formation of B compartment.

However, the role of LLPS in A compartment organization is currently an active area of research. Histone acetylation disrupted compacted chromatin droplets, and multi-bromodomain proteins, such as BRD4, induced LLPS of acetylated histones, forming liquid droplets (30, 31). Growing evidence suggests that phase separation is one of the critical mechanisms of chromatin compartmentalization in 3D genome organization, although further efforts are needed to decipher the dynamics and mechanisms of regulation involving phase separation in chromosome compartmentalization.

TADs and loop domains

Chromosome folding studies with low-resolution (40 kb) Hi-C and chromosome conformation capture carbon copy (5C) data empirically identified highly self-interacting mega-base genomic regions, called TADs (32-34). Later, Rao *et al*. has identified contact domains ranging in size from 40kb to 3 Mb with improved resolution, corresponding to TADs from the initial low-resolution conformation studies. Notably, a large proportion of the contact domains form a chromatin loop, termed as "loop domain". The boundaries of TADs or loop domains are enriched with convergent CCCTC-binding factor (CTCF) motifs, therefore demarcated by CTCFs (Fig.1) (35, 36). These domains are thought to represent stable units of genome regulation, since the boundaries of them or domain interval remain highly stable across distinct cell types and even different species (32, 36, 37). The notion that TADs are highly conserved regardless of cell types and species has been challenged by comparative, finer-resolution Hi-C and single-cell chromosome conformation studies. Eres *et al*. investigated conservation of TADs in induced pluripotent stem cells (iPSCs) from humans and chimpanzees, which revealed that 78 % of

domain intervals and 83% of domain boundaries were shared between the two primates, a much lower portion of genomic information compared with other functional genotypes and phenotypes (37, 38). Moreover, multiple plant species such as maize, tomato, rice and mustard plants showed relatively little conservation of TADs via testing whether orthologous genes are located in a same TAD in different species of plants (37, 39, 40). The sparsity of chromosome conformation data with a finer resolution in diverse species and the lack of adequate analytical tools to directly compare TAD domains and boundaries between different species makes it difficult to draw a clear conclusion of TAD conservation.

In addition to the variability between different species, scHi-C and super-resolution imaging have spotted heterogeneity of TAD structures between individual cells (16, 17, 41). Furthermore, other scHi-C studies revealed TAD domains became visible at the population level, but not constant structures under single-cell resolution, indicating that TAD may be a malleable structure at a single-cell level (42, 43). Investigating the role of TAD variability in genome regulation requires systematic investigation into the heterogeneity and its outcome in gene expression. TADs have been known to serve as a physical barrier to facilitate chromatin interactions, such as enhancer-promoter chromatin looping, within the same TAD while restricting the interactions across different topological domains (3, 44). However, such insulation function of the TAD has been challenged by recent studies in different species including humans, mouse and fly. In humans, capture Hi-C (CHi-C) analysis of 17 primary hematopoietic cell types revealed that long-range interactions often span several TADs (45). The interactions between polycomb-bound regions occasionally involved multiple TADs in mouse embryonic stem cells (ESCs) (46). Additionally, Yokoshi *et al.* tested the role of TADs in living fly embryo via live imaging to monitor the gene expression and identified that a deletion in one of the endogenous ftz-TAD

boundaries resulted in repression of Sex combs reduced (Scr), a gene located in neighboring TAD of ftz-TAD. The enhancer of Scr is located in the flanking TAD of ftz-TAD, the opposite side of the TAD containing Scr, which suggests that Scr skips the ftz-TAD to physically contact its enhancer in the different TAD. The deletion of one of the TAD boundaries of ftz-TAD prevented the interaction between Scr gene promoter and its enhancer, leading to the repression of Scr gene. This result suggests that appropriate topological organization maintained by boundary elements plays a critical role in promoting inter-TAD interaction between enhancers and promoters located in distinct TADs (47, 48). The role of TAD in the communication between promoters and enhancers located in different TADs remains to be elucidated systemically in different biological contexts.

Loop extrusion is a major mechanism contributing to the formation of TAD domains. The loop extrusion model states that structural maintenance of chromosome (SMC) complexes are loaded on to chromatin via cohesin loading factors, Nipped-B-like protein (NIPBL), also known as SCC2, and then travel along chromatin while extruding DNA outward in an ATP-dependent manner. They stall at convergent CTCF motifs, which often create domain boundaries, and can be unloaded from chromatin by wings apart-like protein homologue (WAPL) (Fig.2) (49). This model explains chromatin looping preferentially within a TAD domain, as the loop formation via extrusion mechanism stops at the boundary of TADs. Other studies including single-molecule-imaging technologies further support the loop extrusion model by showing that the cohesin or condensin complex moves along naked DNA molecules *in vitro* (50-54).

Long-range enhancer-promoter contacts

Enhancers are known to mediate spatiotemporal gene expression across distances of kilobase, and even megabases. While TAD domains and boundaries are stable across different cell types at

the population level, chromatin loops between CREs are highly dynamic between different cell

lineages and developmental stages (3, 55). A historically well-known example is the locus

control region (LCR) enhancer, which interacts with β-globin gene in erythrocyte, but the

contact between LCR enhancer and β-globin gene does not exist in other lineages including

neuron where β-globin is not expressed (56-58). The local examination of enhancer-promoter

interactions with 3C or chromosome conformation capture-on-chip (4C) is limited to single or

multiple genomic loci. Genome-wide chromatin-looping dynamics in various biological contexts

has been studied using novel methods for active regulatory element centric chromosome

conformation capture technologies, such as CHi-C and H3K27ac HiChIP (58-63). For example,

Rubin *et al*. demonstrated the cooperation between two types of enhancer-promoter contacts for

adequate gene expression during keratinocyte differentiation using CHi-C: one mediated by

stable enhancers, pre-established in progenitor keratinocytes, and the other involving dynamic

enhancers acquired during differentiation (63).

Besides the architectural proteins, such as CTCF and cohesin complexes, mediators which

frequently co-bind with cohesin at promoters and enhancers, are important in facilitating

enhancer-promoter interactions, as the knockdown of mediator subunits decreased the looping

interaction frequency at mediator and cohesin-loaded loci in mouse ESCs (Fig.1)(64).

Transcription factors (TFs) are also critical players in chromatin looping via CRE binding and

oligomerization (65). Yin Yang1(YY1), a ubiquitously expressed TF, is a well-known player in

chromatin looping, enriched at enhancers and promoters, mediating enhancer-promoter contacts

by dimerization. Depletion of YY1 reduced enhancer-promoter looping frequency and target

gene expression (66). Multiple species of non-coding RNAs, such as enhancer RNA (eRNA) and

long non-coding RNA (lncRNA), also regulate chromatin looping (Fig.1)(67). For instance, an

eRNA transcribed from an enhancer of Bcl11b, facilitates the interaction between the Bcl11b

enhancer and the promoter of Bcl11b by recruiting the cohesin complex to the loci and then

repositioning of the enhancer from lamina to the nuclear interior (68).

Chromatin loops are not limited to enhancer-promoter interactions. Other types of contacts such

as enhancer-enhancer, promoter-promoter in the spatial gene regulatory network exist by

forming clusters of each element. Such clusters have been observed in mouse ESCs, thymocytes,

olfactory sensory systems, and human T cells, and regulate gene expression according to

biological context (46, 61).

TECHNOLOGIES TO STUDY CHROMOSOME ARCHITECTURE

Technological advances in chromatin biology developed in the last few decades have broadened

our understanding of chromosome architecture. 4DN Network and other investigators have

developed various genomics and imaging-based tools (Fig.3 and Table 1) (5). In this section, we

will discuss details of these technologies and related biological findings.

Genomics-based technologies

With the emergence of next-generation sequencing (NGS), chromosome conformation analysis

has been expanded to the genome scale, uncovering principles of genome folding. The

established principles of chromosome folding are being revisited at the single-cell level, although

most of the current studies using genomics approaches are still focused at the population level.

Genomics-based technologies can be classified into two distinct types: 3C-based and ligation-

free methodologies (Fig.3 and Table 1).

3C-based methods

The single long-range interaction between two genomic loci has been detected using 3C-based tools which employ the principle of proximity ligation. Cross-linked and digested DNA fragments in the nucleus are subjected to limited ligation between DNA fragments in the same crosslinked unit, which is favored over ligation of random fragments (69). Using primers targeting two loci of interest, the interaction frequencies can be measured by quantitative polymerase chain reaction (qPCR) (Fig.3). Since the 3C libraries contain all the proximal interactions between crosslinked and fragmented DNAs in the nucleus, they could be easily subjected to a high-throughput analysis of chromatin interactions via microarrays or NGS techniques. The 3C technique was readily expanded to its derivatives such as 4C, 5C and Hi-C (7). The 4C technique is 'one to all' method, as primers targeting a region of interest are used to amplify ligated DNA fragments, and therefore capture all possible chromatin interactions of a single target locus (70). 4C is also used as a validation tool for Hi-C and other 3C-derived genome-wide scale technologies, as it requires a low sequencing depth (1-5 million reads per library) to obtain a detailed view of a locus of interest ('viewpoint') centered interaction maps (60, 61) (Fig.3).

The 5C technique employs multiplexed forward and reverse primer sets for all restricted DNA fragments located in a genomic region of interest spanning few hundred kbs to Mbs, which are ligated via annealing next to each other. The ligated primer pairs are amplified through a universal sequence at the end of each primer and sequenced to provide quantitative information regarding the interactions within the region of interest. The 5C method detects 'many to many' interactions using the multiplexed primer sets. The 5C technique is a cost-effective method used to investigate the interaction between DNA elements in a large genomic region of interest (Fig.3) (71).

In order to overcome the scale limitation of 3C, 4C and 5C, Hi-C was introduced in 2009 (20).

Hi-C maps all genome-wide chromatin interactions from 3C workflow via modification and

incorporation of biotin at the end of restricted DNA fragments before ligation, recovery of all

ligated products by streptavidin pull-down and finally, massively parallel sequencing of the

enriched interactions (Fig.3). One disadvantage of this proximity ligation-based method is that

the ligation step is performed in diluted solution, but not in the nucleus. Thus, the restricted DNA

fragments float freely in the diluted solution and randomly ligate each other, which results in a

high frequency of false interactions. In 2014, *in situ* Hi-C was developed as an improvement

over conventional Hi-C. The major improvement of *in situ* Hi-C is that it can be used for ligation

in the nucleus, thereby preserving the native environment, where the positioning of

chromosomes is intact. Additionally, the *in situ* Hi-C protocol adopts a four-cutter enzyme

instead of a six-cutter restriction enzyme, used in Hi-C, for higher resolution (1 kb) compared

with conventional Hi-C (1Mb) (36). *In situ* Hi-C can be used to generate higher-resolution

contact maps for the detection of chromatin loops in addition to larger chromatin structures, such

as compartments and TADs (7, 36). Many other Hi-C derivatives have been developed to

improve the resolution of conventional Hi-C and *in situ* Hi-C, including Micro-C and DNase Hi-

C (Table 1). Micro-C uses micrococcal nucleases (MNases) instead of six-cutter enzymes in Hi-

C protocol, enabling nucleosome resolution for chromosome contact maps (72). Recently,

Micro-C has been used for mammalian genomes, unveiling novel insights into chromatin

interactions below the level of TADs that are obscure in conventional Hi-C data. Micro-C was

used to capture local chromatin loops at finer scales, 100 bp to 20 kb, in enhancer-promoter or

promoter-promoter interactions mediated by active transcription forming a hub to promote CRE

contacts (Fig.3) (73). Similarly, DNase Hi-C uses deoxyribonuclease I (DNase I) instead of

restriction enzymes used in Hi-C workflow to map the chromatin interactions under higher resolution (Fig.3) (74, 75).

Several factors such as architectural proteins, histone marks and non-coding RNAs are known to facilitate 3D chromatin folding (Fig.1). The 3C-based technologies have been adapted to other methodologies to elucidate specific factor-driven chromatin interaction (Table 1). Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) employs basically a combination of Hi-C and chromatin immunoprecipitation (ChIP) to enrich chromatin contacts mediated by a protein of interest. Despite the enrichment of specific protein-mediated chromatin interactions with ChIP, ChIA-PET requires a large amount (hundreds of millions) of starting material and sequencing reads per library to obtain enough informative reads (62, 76, 77). To overcome these shortcomings of ChIA-PET, HiChIP employs *in situ* Hi-C and bead-based Tn5 library generation strategy, requiring much less material to start with (~1 million mammalian cells depending on proteins of interest) and sequencing reads compared to ChIA-PET (62). Similarly, another method called proximity-ligation-assisted ChIP-seq (PLAC-seq) was developed at the same time to improve the sensitivity and efficiency of ChIA-PET. In PLAC-seq, the order of proximity ligation and chromatin shearing step is switched as in HiChIP, thereby producing a higher number of useful sequencing reads from fewer cells (Fig.3) (78).

Conventional Hi-C or other similar methods require billions of sequencing reads to achieve a high- resolution map of interactions. To obtain a comprehensive map of enhancer-promoter interactions, Capture-C and CHi-C use oligonucleotide probes, which can be hybridized to genomic regions of interest to enrich contacts containing those target regions (Fig.3) (59, 79). Besides the architectural proteins, RNA has recently gained much attention as an important player in 3D chromosome conformation (67). Compared with the diversity of tools to interrogate

14

protein factor-mediated chromatin contacts, the technologies to analyze genome-wide RNA-mediated chromatin contact maps need further development via high-throughput approaches. HiChIRP was recently developed to examine chromosome conformation mediated by a specific RNA species, which switch ChIP step in the HiChIP protocol with RNA pulldown using biotinylated probes (80). HiChIRP has a few limitations such as targeting a single known RNA at a time, producing one-to-all type of interactions and incapable of investigating chromatin interactions mediated by unknown RNA. Investigation of RNA-associated chromosome conformation requires the development of new tools to uncover the role of diverse RNA species in chromatin interaction.

Conventional Hi-C revealed the hierarchy in chromosome folding in the nucleus and multiple Hi-C-derived methods have improved resolution, and thereby allowed examination of smaller-scaled chromosome conformations including chromatin loops. However, these methods highlight higher-order chromatin architecture only at the population level and cannot address heterogeneity of chromatin folding between individual cells. To investigate the variability of chromosome conformation between individual cells, several new technologies adapting Hi-C such as scHi-C, which is the first method for single-cell analysis to explore genome folding at a single-cell resolution have been developed (Fig.3 and Table 1). Fragmentation and ligation steps in scHi-C are performed in a population of the nucleus, and the individual nuclei are selected under the microscope (Fig.3) (16). Since scHi-C requires laborious physical separation of the nuclei to obtain individual nuclei, the method is difficult to be used in a large-scale analysis. To simplify the procedure for efficient analysis of single nuclei, the single-cell combinatorial indexed Hi-C (sciHi-C) uses combinatorial cellular indexing, previously used in single-cell RNA, ChIP and ATAC-seq, to achieve contact maps in single-cell resolution (Fig.3) (81-86).

Similarly, Dip-C developed in 2017, leveraged multiplex end-tagging amplification (META) to increase DNA recovery and constructed a 20 kb contact matrix for each parental haplotype (43). Recently, two studies suggested tools to investigate single-cell chromosome conformation and DNA methylation status concurrently (Table 1). Single-cell methyl Hi-C (scMethyl-HiC) and single-nucleus methyl-3C sequencing (sn-m3C-seq) combined *in situ* Hi-C followed by fluorescence-activated cell or nuclei sorting and whole-genome bisulfite sequencing (WGBS). These methods added another layer of information correlating chromosome conformation with DNA methylation status at the sites of interaction, revealing inverse correlation between methylation of CTCF sites at the site of interaction with the frequency of chromatin interactions (13, 14).

Ligation-free methods

All the 3C-based approaches involve a ligation step to connect the ends of DNA fragments in the same crosslinked cluster. Ligation links one end to another end, producing 1:1 ligated DNA, and therefore intrinsically dilutes the complex interactions between multiple DNA elements in the native environment of the nucleus (6, 7). To capture all the dynamic chromatin interactions, several ligation-free approaches have been developed: GAM, split-pool recognition of interactions by tag extension (SPRITE), and chromatin-interaction analysis via droplet-based and barcode-linked sequencing (ChIA-Drop) (Fig.3 and Table 1). GAM is the first genome-wide method to capture all possible contacts between any genomic loci without ligation process, which produces a contact map including multivalent chromatin interactions. To gain the information of multivalent chromatin contacts, all the DNA elements in a large number of randomly selected thin nuclear cryosections are sequenced to calculate co-segregation frequencies between every pair of genomic regions or triplets. A new mathematical model, called

statistical inference of co-segregation (SLICE) was invented simultaneously to identify specific interactions from the measured co-segregation frequencies. The capacity of detecting triple contacts of GAM enabled the identification of abundant three-way interactions among super-enhancer-containing TADs which span tens of Mbs (Fig.3) (8). SPRITE repeats split-pool barcoding of crosslinked and fragmented chromatin followed by sequencing and identifies contacts by matching all the reads carrying identical barcodes. In this way, multiple fragments in a same crosslinked complex which contain a same set of unique ligated tags can be identified and inferred as multiple DNA interactions (Fig.3).   The higher order complex chromatin interactions such as contacts between A compartments have been observed due to the capacity of detecting multiple chromatin contacts concurrently (87). ChIA-Drop leverages microfluidics to deliver a unique barcode to each crosslinked and fragmented chromatin complex loaded onto a droplet, and thereby provides single-molecule precision (Fig.3) (88).

It has been known that nuclear structures act as scaffolds for chromosome folding, and therefore the different compartments are associated with distinct parts of nuclear bodies (4).

TSA-seq is another ligation-free method used to measure cytological distances of genomic regions relative to a particular nuclear structure including nuclear speckles via tyramide-signal amplification (TSA), a widely used technique in immunocytochemistry (Table 1) (89). TSA uses antibody-conjugated horseradish peroxidase (HRP) that binds to a specific protein in the nuclear compartment, where it catalyzes the formation of biotin-tyramide free radicals, which diffuse and bind to nearby genomic regions. The biotin-marked genomic DNA is collected via biotin pull down and is subjected to sequencing to analyze regions close to the protein, a component of a specific nuclear structure (90). Recently, an upgraded version of TSA-seq has been used for different human cell types including ESC, fibroblasts, erythroleukemia, and colon carcinoma, to

detect high levels of conservation of genome organization relative to nuclear speckles between the different cell types. This result suggests an important role of nuclear speckles as a scaffold in chromosome folding (91).

The ligation-free methods described above successfully demonstrated their advantages over ligation-based methods in identifying multi-contacts between genomic loci, but limitations do exist for each technology.

GAM and ChIA-Drop requires special instruments for cryosectioning and microfluidics respectively, which makes it difficult to apply these techniques if a laboratory without the instruments. Although SPRITE is one of the ligation free methods, it still depends on ligation of an oligo tag to each fragment end in the interacting cluster which demands high efficiency of fragmentation step to make the fragment end available for ligation of an oligo tag.

Imaging-based technologies

Although genomics-based technologies have expanded our understanding of higher-order chromatin organization, they are limited to the study of pairs of genomic regions, without disclosing the direct spatial position of each region in the nucleus. Furthermore, despite the recent developments and improvements in single-cell genomics-based approaches, it is still challenging to obtain chromatin interaction map at the single-cell level. In contrast, imaging-based technologies can be used to immediately visualize the exact spatial position of genomic loci at single-cell resolution. Recent efforts focusing on increasing the throughput of imaging tools encourage us to apply those to investigate genome-wide chromatin interactions. In this section, we will elaborate two types of imaging-based approaches used to examine chromatin interactions: FISH-based technique and live-cell imaging (Fig.3 and Table 1).

FISH-based technique

DNA FISH is a well-established technique to visualize chromatin contacts in fixed cells (92).

DNA FISH is traditionally used to measure distances between two or more loci with different

fluorescent labels, and can be adapted to other derivatives, including 3D FISH which applies

FISH to whole cells or tissue and cryo-FISH using thin cryosections of cells (Fig.3 and Table

1)(7).

However, traditional DNA FISH is incapable of resolving individual loci when multiple genomic

regions are visualized concurrently due to the limited number of fluorescent colors marking each

locus differentially, and diffraction issues. Therefore, it is difficult to decipher complex

chromatin contacts. Furthermore, traditional DNA FISH uses a set of DNA fragments as oligo

probes ranging in size from 150 to 500 bp, hybridized to genome regions ranging in length from

30 kb up to a few hundred kbs, making it challenging to map fine-scale chromosome

conformations, such as enhancer-promoter loops that often occur at less than 100 kb distance (7,

9).

To address these issues, chromatin tracing, a highly multiplexed DNA FISH was introduced in

2016, which enabled direct tracking and visualizing of chromosome folding path (Fig.3 and

Table 1)(11, 12, 93). Recent development of a tool to massively synthesize oligo probes with

short length (~60-100 bp) and high specificity in parallel, called Oligopaints, has facilitated

chromatin tracing (9, 94). Chromatin tracing has integrated FISH using diverse florescence oligo

probes produced by Oligopaints and high-throughput imaging tools to pinpoint multiple targeted

genomic regions along the same chromosome, eventually connecting these loci to reveal the 3D

folding path (Fig.3) (10-12, 15). To enhance the accuracy of visualizing each locus, chromatin

tracing frequently employs sequential imaging using a dual-oligonucleotide version of

Oligopaints (9, 95). Primary probes consist of complementary genomic sequence to target loci

and non-genomic sequences known as MainStreet enabling multiple functions such as providing binding sites for secondary probes, amplification and multiplexing with unique barcodes. They are first hybridized to genomic regions of interest. Next, secondary probes are sequentially hybridized to the MainStreet of the primary probes, thereby pinpointing a target region of interest as a single spot with nanoscale accuracy. The process of hybridization and imaging is repeated multiple times and the images are gathered to reconstruct the 3D folding path of chromatin (Fig.3) (11). The first chromatin tracing study targeted tens of TADs in cultured human cells revealing the 3D chromatin folding at TAD-to-chromosome scale (12). The resolution, genomic coverage and the throughput of chromatin tracing have been markedly improved in recent years. For instance, Bintu *et al*. identified sub-TAD structures and the high variability between TADs at 30-kb resolution. Strikingly, this study also revealed that cohesin depletion did not change TAD-like structures at the single-cell level, indicating the role of other mechanisms or players in maintaining the domains (10). Optical reconstruction of chromatin architecture (ORCA), a method combining chromatin tracing and RNA FISH to visualize DNA and RNA simultaneously, reconstructed conformation of the bithorax complex at 2-10 kb resolution to detect the enhancer-promoter contacts in Drosophila embryos (96). Chromatin tracing has been applied to other organisms besides human and fruit flies including *C. elegans* and mouse at ~10 kb resolution, revealing species-specific chromosome conformation (97, 98). Recently, a high-throughput genome-scale chromatin tracing approach, known as multiplexed error-robust fluorescence in situ hybridization (MERFISH), enabled simultaneous imaging of >1000 genomic loci with transcription in individual cells, identifying trans-chromosomal interactions correlating with active transcription (15). This approach improved the scale of

chromatin tracing remarkably and facilitated multimodal analysis of chromatin conformation, transcription, and nuclear structure via sequential imaging of each component (15).

Live-cell imaging

Although FISH-based approaches have been used to reveal the fine-resolution chromatin architecture including enhancer-promoter contacts, such methods are limited to fixed cells, and are incapable of elucidating the spatiotemporal genome dynamics. The emergence of genome editing technologies, such as clustered regularly interspaced short palindromic repeats (CRISPR), have revolutionized a variety of technologies in biomedical research including the imaging tools especially for chromatin interaction to target specific genomic loci of interest in live cells (7, 99). Chen *et al*. first introduced CRISPR applied imaging technology in live cells by tagging the enhanced green fluorescent protein (EGFP) to endonuclease-deficient Cas9 (dCas9) protein, which is recruited to specific loci targeted by small guide (sg) RNA (100). This method has been adapted to other derivatives using dCas9 orthologs tagged with florescent proteins of different colors, facilitating visualization of multiple loci and the distances separating them (Fig.3) (101, 102). Besides dCas9, sgRNAs were modified by fusing the scaffold with viral RNA-recruiting viral proteins tagged with fluorescence, allowing multi-color imaging of several genomic loci at once (102-105). These approaches are useful when targeting repetitive sequences in the genome by amplifying the fluorescence signal easily but are not suitable for targeting non-repetitive genomic regions incapable of amplifying the signal strongly to enable detection. CRISPR-Tag and chimeric array of gRNA oligonucleotides (CARGO) have been developed in 2018 to enhance the signal of dCas9-sgRNA complexes at the non-repetitive genomic loci (Fig.3 and Table 1)(99). CRISPR-Tag is a type of DNA tags that assembles two to six repeats of CRISPR target from *C.elegans,* which have been knocked into specific human protein-coding

genes and recruited dCas9-GFP proteins for imaging (Fig.3) (106). CARGO enables the delivery of 12 guide RNAs cloned in a single plasmid into a single cell. This tool has been used to target 2 kb region of CREs, revealing their dynamics in live ESC (Fig.3) (107).

CONCLUSIONS AND FUTURE DIRECTIONS

Our understanding of 4D genome has been expanded substantially with the development of new technologies unveiling the biophysical and molecular insights for temporal and spatial organization of chromosomes. Recent advances in genomics-based approaches at finer and single-cell resolution provide unprecedented knowledge of the heterogeneity and dynamics of genome architecture concurrently with other epigenomic information such as DNA methylation. The potential of imaging technologies has been revolutionized by multiplexing with the development of Oligopaints and MERFISH, which enables imaging of more than 1000 genomic loci at a time for FISH-based approaches, whereas live-cell imaging which integrates genome-editing technologies with super-resolution imaging can currently target 12 loci at most. Additional efforts to improve resolution and throughput of these genomics and imaging-based tools are needed to unravel the complete mechanisms and identify novel players in chromosome folding at each genomic scale. Moreover, single-cell tools to assay 4D genome with other omics features such as transcriptomics, epigenomics and even proteomics simultaneously can be powerful approaches to understand the role of spatial organization of genome in regulating the genome function precisely at the right time and place.

Since 4DN Network has been launched, the collaborative work has developed diverse new tools summarized in this review and analyzed them systematically in phase 1. The phase 2 was started recently focusing on real-time chromatin dynamics, data integration with modeling, and 4DN function in human health and disease. Ultimately, the collective efforts are expected to identify

novel mechanisms of genome folding and its function in gene regulation, which will expedite

applications of the knowledge into disease diagnosis and medicine development in the future.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Figure1. An overview of hierarchical organization of eukaryotic genome.

Genome is hierarchically packed into a tiny nucleus at different scales: chromosome territories,

compartments, topologically associating domains (TADs) and chromatin loops. Chromosomes

occupy their own preferential location in the nucleus (multiple colors), referred to as territories.

Each territory consists of two compartments: A and B. A compartment (light yellow) is

composed of active epigenetic marks and actively transcribed genes and associated with nuclear

speckles (red circles). However, repressive epigenetic marks and inactive genes constitute the B

compartment (light blue), located close to the nuclear lamina (yellow and red wavy lines) and

nucleolus (black circle). TADs are defined as highly self-interacting domains with the

boundaries demarcated by CCCTC-binding factors (CTCFs) (light red) with cohesin complex

(blue) (middle square). CTCF and cohesin (blue) play an important role in loop extrusion

mechanism. At the finest scale of genome folding, chromatin looping is mediated by multiple

different factors such as transcription factors (blue-green), YY1 (salmon), mediators (green), RNA polymerase II (pink) and non-coding RNAs (wavy brown lines) that promote contacts between cis-regulatory elements (CREs) (light yellow and orange square representing enhancer and promoter, respectively) (right square). The size of each scale ranges from 1 to 100 Mb for territories and compartments, 40 kb to 3 Mb for TADs and loop domains, and from 1 kb to few Mb for long-range enhancer-promoter contacts.

Figure2. Loop extrusion mechanism. Ring-shaped cohesin complex (blue) is loaded on to chromatin via cohesin-loading factors, such as NIPBL (dark orange). The cohesin complex slides the chromatin string till it encounters convergent CTCF motifs (red triangles), while it extrudes the chromatin outward through the ring-shaped structure. The cohesin-unloading factor, WAPL (purple) unloads the cohesin from the chromatin, recycling the cohesin complexes.

Figure3. Technologies for studying 4D nucleome. Technologies for 4D nucleome research are classified into two types: genomics and imaging-based methodologies. 3C-based and ligation-free methods are the major two types of genomics-based tools. Imaging-based tools are further classified into FISH-based methods and live-cell imaging. A main principle and workflow are demonstrated in the figure for each method (written in bold letters).

For 3C-based methodologies, modifications of 3C workflow are depicted or described with the corresponding method in colored boxes. The location of method indicates where the modification has been applied in 3C workflow. For example, DNase-Hi-C uses DNase for fragmentation instead of a six or four cutter enzyme, therefore located under 'Crosslinking' step, indicating that the modification is applied after the crosslinking step. Downstream applications are indicated with the method via color-coded squares such as blue square for PCR, green square for microarray and orange square for sequencing. In addition, the scale of each method is shown

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Chromosome territories and compartments

TADs and loop domains

**Long-range enhancer-promoter contacts**

Scale

1-100 Mb

40 kb - 3 Mb

1 kb - few Mb

Chromosome territory

Nuclear lamina

Nuclear speckle

Nucelolus

A Compartment   B Compartment

CTCF
Cohesin
TAD
Loop domain

YY1   Mediator   TF   RNA pol II
Enhancer   Promoter
eRNA
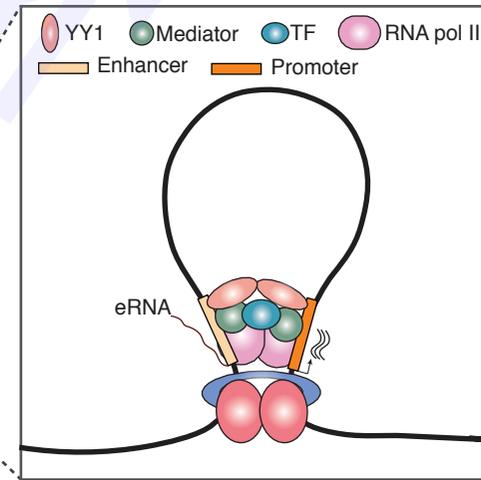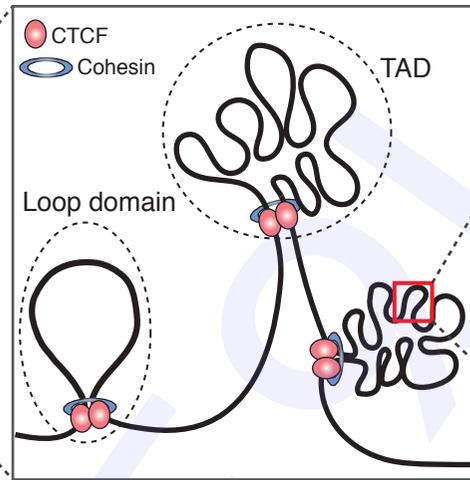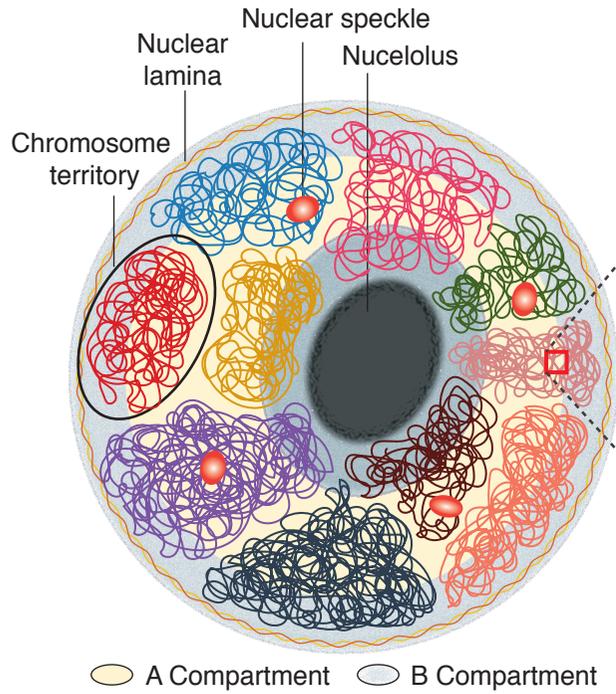
- CTCF
- Cohesin complex
- Forward CTCF motif
- Reverse CTCF motif

NIPBL

NIPBL

WAPL

WAPL
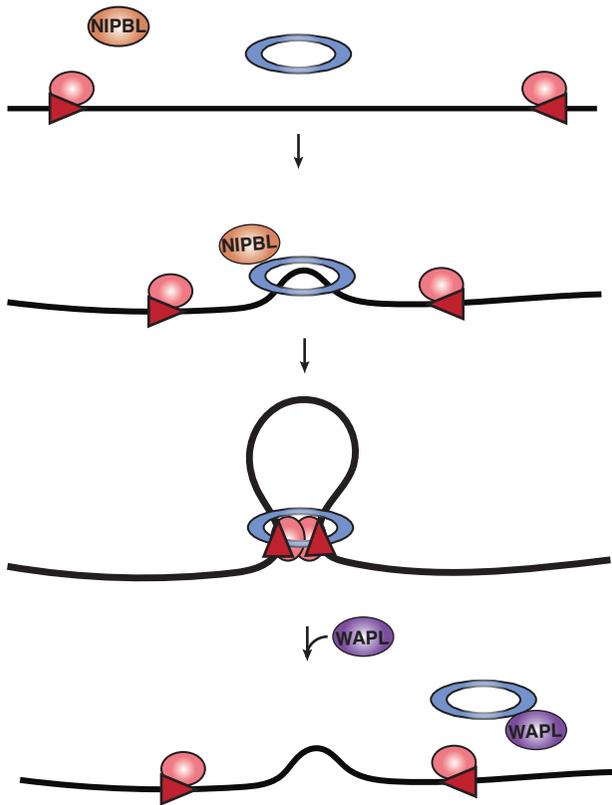
Genomics

3C-based

Downstream application: ■ PCR ■ Microarray ■ Sequencing

Crosslinking
Fragmentation
Proximity - ligation
Reverse Crosslinking

DNase/MNase
DNase Hi-C
Micro-C ■

ChIA-PET ■
sciHi-C ■
combinatorial-multiplexing

HiChIP ■
PLAC-seq ■
scHi-C ■
manual-isolation

3C ■
4C ■ ■
5C ■ T7 T3
Hi-C ■
CHi-C ■
Capture-C

Population
Single-cell

Ligation-free

GAM
Cryosectioning & laser microdissection
Slice1
Slice3
Slice2

Calculate cosegregation frequency

Slice1 + + − − −
Slice2 − − + + −
Slice3 − − − − +
Slice #n

SPRITE
Crosslinked & fragmented chromatin
Split & Barcode Repeat X5
Pool
Identify segments in a same cluster

ChIA-DROP
Gel beads with barcodes
GEM droplet
Microfluidics device
Identify segments in a same cluster

Imaging

FISH-based

Fixation & DNA denaturation

3D-FISH   **cryo-FISH**
Cryosection

Fluorescent-labeled probes

Imaging

Chromatin tracing
Primary probes
Hybridization 0
Secondary probes
Hybridization 1
Bleached probes
Hybridization 2
Hybridization 3
Genomic DNA

Reconstruct chromatin trace

Live-cell imaging

CRISPR-TAG
Repeat units
C. elegans CRISPR target sites
Knock-in
Live-cell + dCas9-eGFP
Imaging

CARGO
■ target cis-regulatory elements

Table 1. Technologies for mapping 4D genome

Class	Assay	Bias	Scale	Resolution
Genomics	**3C-based**			
	3C	Specific-primer target	One vs one	Population
	4C	Specific-primer target	One vs all	Population
	5C	Specific-primer target	Many vs many	Population
	Hi-C	None	All vs all	Population
	in situ Hi-C	None	All vs all	Population
	Micro-C	None	All vs all	Population
	DNase Hi-C	None	All vs all	Population
	ChIA-PET	Specific-protein mediated	Many vs all	Population
	PLAC-seq		Many vs all	Population
	HiChIP		Many vs all	Population
	Capture-C/ C-HiC	Specific-DNA elements involved	Many vs all	Population
	HiChIRP	Specific-RNA mediated	Many vs all	Population
	scHiC	None	All vs all	Single cell
	sciHiC	None	All vs all	Single cell
	Dip-C	None	All vs all	Single cell
	Ligation-free			
	GAM	None	All vs all	Single cell
	SPRITE	None	All vs all	Population
	ChIA-DROP	None	All vs all, Many vs all	Population
	TSA-seq	Nuclear-structure centric	Many (nuclear compartment) vs many (genomic loci)	Population
Imaging	**FISH-based**			
	3D FISH, Cryo-FISH	Specific-probe target	2-52 regions	Single cell
	Chromatin tracing	Specific-probe target	>1000 genomic loci	Single cell
	Live-cell imaging			
	CRISPR-Tag	CRISPR-Tag target	One specific locus	Single cell
	CARGO	Multiplexed gRNA target	12 loci	Single cell