Single-cell Hi-C bridges microscopy and genome-wide sequencing approaches to study 3D chromatin organization

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Recent years have witnessed an explosion of the single-cell biochemical toolbox including chromosome conformation capture (3C)-based methods that provide novel insights into chromatin spatial organization in individual cells. The observations made with these techniques revealed that topologically associating domains emerge from cell population averages and do not exist as static structures in individual cells. Stochastic nature of the genome folding is likely to be biologically relevant and may reflect the ability of chromatin fibers to adopt a number of alternative configurations, some of which could be transiently stabilized and serve regulatory purposes. Single-cell Hi-C approaches provide an opportunity to analyze chromatin folding in rare cell types such as stem cells, tumor progenitors, oocytes, and totipotent cells, contributing to a deeper understanding of basic mechanisms in development and disease. Here, we review key findings of single-cell Hi-C and discuss possible biological reasons and consequences of the inferred dynamic chromatin spatial organization.

Keywords:
chromatin; chromatin compartment; CTCF/cohesin-anchored loop; genome spatial organization; single-cell Hi-C; TAD

DOI 10.1002/bies.201700104

Introduction

How the 2 m of DNA fit into a 10 μm sized nucleus has been a fundamental mystery in biology for decades. The spatial organization of chromatin fibers can be studied using microscopy and biochemical approaches [1]. Although microscopic approaches focus on individual cells and, in the case of live-cell imaging, can document dynamics, biochemical techniques reveal characteristics of the cell population average. The advantage of biochemical studies is the possibility of performing genome-wide analysis above and beyond the analysis of only a few selected genomic loci by microscopy.

The development of fluorescence techniques such as fluorescence in situ hybridization (FISH) and in vivo imaging with fluorescent proteins have opened an opportunity to track the behavior of certain genomic loci or whole chromosomes. Chromosome territories (CTs) [2] composed of chromatin globular domains [3], largely non-random positioning of gene loci within the CTs [4], a spatial proximity between certain enhancers and promoters [5], and spatial associations between remote gene loci at transcription factories [6, 7], and nuclear bodies [8, 9] were discovered using FISH. Optical imaging of living cells harboring chimeric proteins tagged by GFP or other fluorophores demonstrated a rapid movement of gene loci within the cell nucleus [10, 11]. However, the small number of fluorophores, and relatively low resolution of even super-resolution microscopy, limit the use of FISH for high-throughput studying fine-scale chromatin spatial organization.

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Abbreviations:
3C, chromosome conformation capture; **CT,** chromosome territory; **ES,** embryonic stem cells; **FISH,** fluorescence in situ hybridization; **Hi-C,** high-throughput chromosome conformation capture assay; **iPSCs,** induced pluripotent stem cells; **kb,** kilobases thousands of base pairs; **LAD,** lamina-associated domain; **NAD,** nucleolus-associated domain; **scHi-C,** single-cell Hi-C; **snHi-C,** single-nucleus Hi-C; **TAD,** topologically associating domain.

The biochemical technique known as Chromosome Conformation Capture (3C) [12] and high-throughput derivatives of 3C [13] uncovered general principles of the genome folding in a variety of taxa from bacteria to humans [14]. The mammalian interphase genome was found to be hierarchically folded at multiple levels. The whole chromosome possesses features of a fractal globule partitioned into active A- and repressed B-compartments [15]. At the megabase scale, chromatin is folded into topologically associating domains (TADs) representing genome regions enriched with intra-domain interactions and commonly interpreted as chromatin globules [16]. Construction of high-resolution chromatin interaction maps revealed that chromatin within some TADs is organized into consecutive CTCF/cohesin-anchored loops of various lengths, and some of these loops are formed between enhancers and promoters [17]. All conventional 3C-based methods require 10^5–10^7 of cells for an experiment. Although compartments, TADs, and loops were found in the analysis of population data and thus may represent a population average, functional analysis strongly suggests that these levels of chromatin folding are biologically relevant. Accordingly, it has been observed that fusion of TADs due to the deletion of a boundary region results in deregulation of gene expression [18]. On the other hand, studies performed using microscopy approaches [1] and available single-cell biochemical techniques, such as single-cell RNA-seq [19], ATAC-seq [20, 21], and DNA-methylation analysis [22], show that the mode of chromatin folding, the patterns of epigenetic modifications and the transcriptome characteristics may vary considerably in different cells of the same population. As discussed below, this variability is thought to be biologically relevant. Studies of individual cells instead of cells populations are likely to enable the identification and characterization of rare cell subtypes present in a population [23–25], including cells that can give rise to tumors. That is why single-cell biochemical studies are currently considered as a "trend of a time." Here, we discuss current advances in single-cell Hi-C studies providing complementary information to FISH and population-based analysis of the genome spatial organization (Fig. 1). We address both the technical issues and the significance of these studies for disclosing the role of chromatin fiber dynamics in major genetic processes.

Single-cell maps of the genome topology: Where are we now?

Single-cell Hi-C is a fast moving field with currently six papers on Hi-C experiments and technical protocols [26–31] (Fig. 2). Pioneering work of Peter Fraser's laboratory provided the first whole-genome map of chromatin spatial structure in a single cell [26]. The authors applied a conventional Hi-C protocol that includes fragmentation of chromatin with a restriction enzyme, DNA-end biotinylation, and subsequent blunt-end ligation of closely located chromatin fragments within the cell nucleus [15]. The key technical challenge of this work is manual isolation of dozens of single somatic nuclei after the ligation step followed by the pulling down of biotinylated ligation junctions from each isolated nucleus separately. The obtained Hi-C data enabled the analysis of whole-chromosome configuration; TADs were inferred to exist in single cells albeit the number of captured contacts (about 10^4 per cell) was not sufficiently high to study these fine-scale features of the genome topology. The main finding of this work is a prominent cell-to-cell variability in the chromosome spatial organization revealed by Hi-C maps and Hi-C-data-based structural modelling of the X chromosome. The spectrum of both inter- and intra-chromosomal contacts of X chromosome domains was found to be considerably different in various cells, possibly reflecting a naturally flexible and dynamic structure of a whole chromosome possessing features of a fractal globule [32].

On the other hand, X chromosome configurations derived from different cells demonstrated certain common structural features, such as preferential location of a subset of the extended genomic domains on the surface of the chromosome territory. Importantly, both transcriptionally active and repressed lamina-associated domains (LADs) are present among those located at the surface of chromosome. The biological significance of such organization is generally clear. Active regions benefit from the location near the chromosome surface being exhibited into interchromatin compartment enriched with transcription activators and facilitating RNA transport [33], and LADs gain access to the nuclear lamina. In this regard, the results of single-cell Lamin-DamID experiments are of particular interest [34]. In mammalian cells, about 34% of lamina-associated genomic regions show considerable cell-to-cell variability in their contacts with the nuclear lamina. Contacts of neighboring loci are highly coordinated and such cooperativity is partly linked to the spatial proximity between the loci. Apparently, the fact that a chromosome adopts a wide set of alternative configurations in a cell population could be partly linked to a cell-to-cell variability in a number/spectrum of LADs from this chromosome associated with the nuclear lamina. The main question here is which one comes first: different chromosome configurations allow different LAD subsets to be juxtaposed to the lamina, or alternative LAD interactions with the lamina dictate (to some extent) the overall folding pattern of their host chromosome? We suppose that the existence of about 15% of constitutive LADs associated with the lamina in more than 80% of cells [34] may restrict the number of enabled chromosome conformations and thus contribute to the underlying chromosome configuration.

Marked variation of the overall chromosome spatial structure among different cells was also observed in another study that utilized single-cell Hi-C data from haploid cells (thus, these data are single-nuclei, in fact) for the structural modelling of the whole genome with resolution of 100 kb [29]. Strikingly, systematic analysis of the calculated 3D structures showed that TADs from both A- and B-compartments can adopt a wide and virtually continuous repertoire of spatial configurations from a completely condensed globule to a highly extended stretch. This observation makes it possible to draw two important conclusions concerning chromatin spatial dynamics: (i) the idea that a TAD represents relatively stable chromatin globule in a living cell is a convenient but considerably oversimplified assumption, and (ii) active and repressed TADs in mammalian cells appear to differ

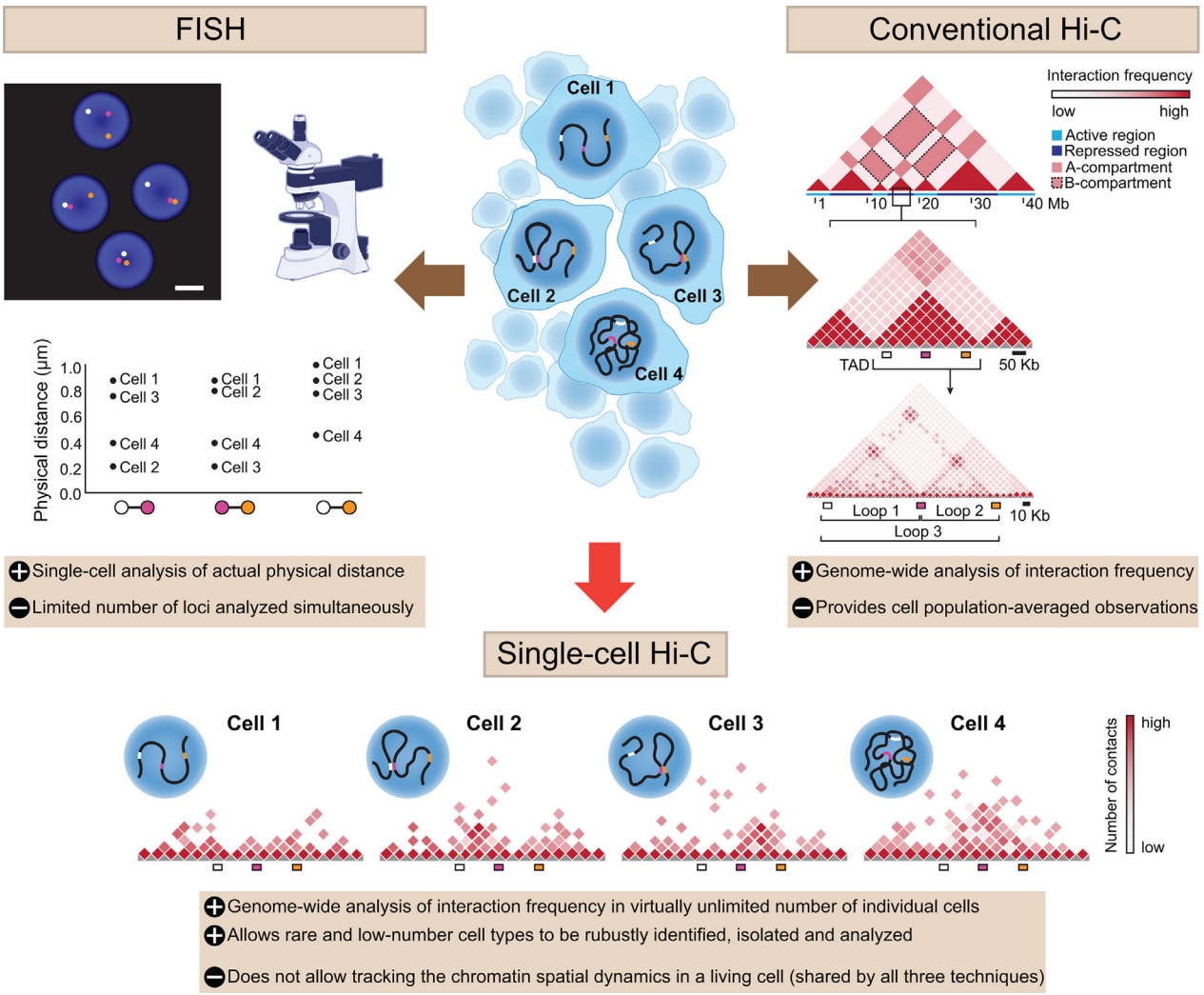


Figure 1. FISH, conventional Hi-C and single-cell Hi-C provide complementary information on chromatin spatial organization. While FISH allows one to measure an actual physical distance between a limited number of loci, and conventional Hi-C provides an integrated information on the chromatin topology in a cell population, single-cell Hi-C data represent a snapshot of the chromatin spatial organization in an individual cell. White, magenta and orange colored short lines and circles on all panels represent three certain genomic regions. Left panel shows illustrative representation of FISH results; right and bottom panels show illustrative conventional and single-cell Hi-C maps, respectively.

quantitatively rather than qualitatively in terms of chromatin density. The last is in good agreement with the results of diffusion coefficient measurements within interphase and mitotic chromatin, which is apparently even more condensed than heterochromatin in interphase, showing that large molecules diffuse through open and closed chromatin with comparable diffusion coefficients, apparently due to local nucleosome dynamics [35]. Similar to a TAD in a globular state, a given CTCF/cohesin-mediated loop could be found only in a portion of cells in the population. This fact may reflect a stochastic nature of loop extrusion (see section "What mechanisms determine the spatial organization of chromosomes?") that has been extensively proposed as a major mechanism of loop formation in mammals [36, 37]. On the other hand, about 30% of loops bring enhancers and promoters together [17]. Because promoters are active in a discontinuous manner and a spatial proximity to an enhancer is a prerequisite for their activation, a proportion of cell-to-cell variability in a loop profile could potentially underlie transcription pulses.

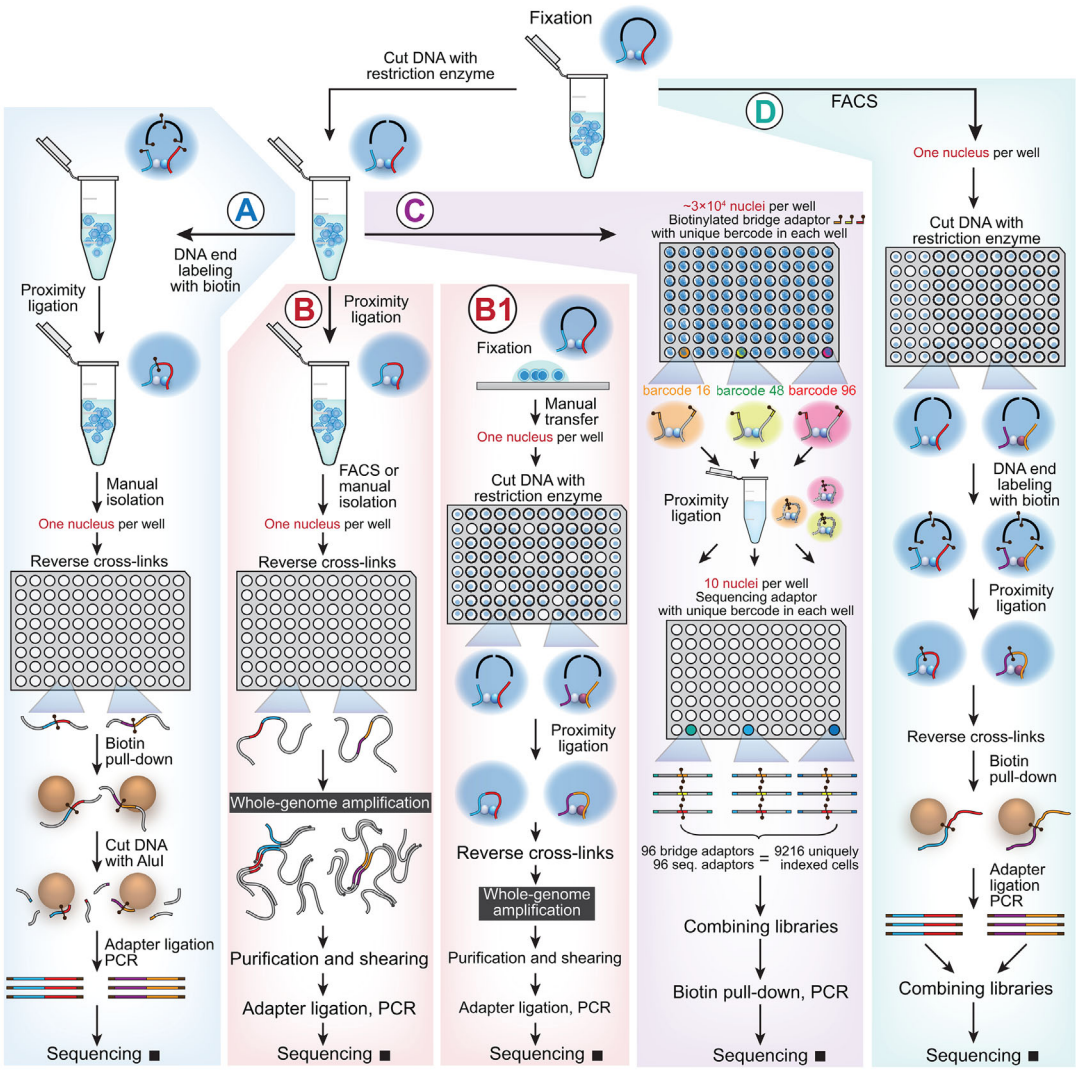
Apart from the observation of heterogeneity of the chromatin folding in a cell population, single-cell Hi-C data provide unexpected advances in disclosing general principles of the genome topology that cannot be uncovered in a conventional population-based Hi-C analysis. For example, a recently published high-throughput single-cell Hi-C analysis [30] reports the presence of a continuous spectrum of inter-TAD contact classes representing a fluent transition of the chromatin states between A- and B-compartment. Furthermore, single-cell Hi-C studies have revised our understanding of TADs as manifestations of chromatin configurations in single cells [31], which will be discussed in the next section.

A

Protocol	Strategy	Cell type, ploidy	Number of captured contacts per cell	
			Median*	Maximum*
Nagano *et al.* 2013 (ref. 26) Nagano *et al.* 2015 (ref. 27)	A	Diploid mouse CD4⁺ T cells	18,166	30,671
Nagano *et al.* 2017 (ref. 30)	A1**	Haploid and diploid mouse ESC	127,233	~500,000
Flyamer *et al.* 2017 (ref. 31)	B B1	Human K562 cells, 2n/2c Mouse oocytes, 4n/2c Mouse zygotes, 1n/1c	182,950 213,533 131,384	356,255 1,906,436 621,074
Ramani *et al.* 2017 (ref. 28)	C	Diploid mouse embr. fibroblasts Diploid human cell lines HeLa S3, K562, GM12878 Haploid human cell line HAP1	~10,000	~100,000
Stevens *et al.* 2017 (ref. 29)	D	Haploid mouse ESC	74,885	122,475

*based on data presented in the paper and supplemental material

B



A1** In this strategy, flow cytometry sorting is used insted of manual nucleus isolation. Also, one-step preparation of library templates with Tn5-transposase tagmentation substitutes AluI digestion, end repair, A-tailing and adapter ligation

Figure 2. Comparison of published strategies for single-cell Hi-C analysis. **A:** A table summarizing key technical features from all papers on single-cell Hi-C published to date. Numbers of captured contacts are extracted from the main text of the papers and from supplemental material, without re-analyzing the original data. **B:** A schematic representation of experimental strategies for single-cell Hi-C. Some routine steps of library preparation such as DNA end repair and A-tailing are omitted.

Single-cell Hi-C allows studying chromatin spatial organization in rare cell types

Single-cell Hi-C provides a possibility for the identification of cell subpopulations in a mixed sample [28] and the potential to study genome folding in rare cell types or indeed in cells that cannot be obtained in numbers sufficient for a conventional Hi-C experiment. Recently, we developed an improved version of single-nucleus Hi-C (snHi-C) to analyze chromatin organization in mouse oocytes and zygotes [31]. We simplified the Hi-C protocol by removing the DNA-end biotinylation step and used phi29 DNA polymerase for the isothermal amplification of genetic material from single nuclei. These technical improvements allowed us to increase the proximity ligation efficiency and chromatin contact retrieval for up to 1–2 orders of magnitude as compared to the first single-cell Hi-C method [26]. Notably, in our best oocyte library, we observe nearly two million unique contacts. These high-coverage data allowed us to track both local and global changes of the genome folding patterns during the oocyte-to-zygote transition. Importantly, we found that A/B-compartmentalization is notably absent in maternal pronuclei, which was also confirmed by FISH with multiple probes for both compartments and subsequently observed in recently published low-input Hi-C analyses [38, 39]. This is the first example of interphase nuclei without spatial segregation of A- and B-type chromatin loci. We propose that such unusual chromatin configuration in maternal pronuclei might reflect a transitional "ground state" providing the basis for formation of a totipotent cell.

Further, snHi-C identified TADs and loops when averaged over all genomic positions in transcriptionally active immature oocytes and in transcriptionally inactive mature oocytes and both pronuclei of zygotes. This is particularly remarkable in the case of zygotes, which are transcriptionally inactive at this stage, suggesting that transcription is not essential for loop extrusion. Moreover, megabase-sized TADs are clearly evident as contact clusters near the diagonal when the best heat maps at resolution of 40 kb are visually inspected. While Stevens et al. demonstrated that a certain TAD adopts a number of spatial conformations in the cell population [29], we show that the contact cluster profile is not fully conserved across the analyzed cells. Contact clusters frequently violate population-identified TAD boundaries, highlighting stochasticity of chromatin folding in a living cell. However, contact cluster profiles from several cells average into a "normal" TAD profile seen in bulk Hi-C data of ES cells when pooled together (that was also observed in [26]). We consider it likely that contact clusters are single-cell manifestations of loop extrusion in diverse conformations and states.

What mechanisms determine the spatial organization of chromosomes?

The above-discussed data denote the presence of a large degree of stochasticity in the chromatin spatial organization at the levels of loops, TADs, and whole chromosomes. The stochasticity is inherent to macromolecular systems. It is determined by thermal motion and conformational flexibility of macromolecules. Computer modeling demonstrates that chromatin fibers can adopt a number of alternative configurations that closely match each other as far as the thermodynamic parameters are considered [40]. The key question is thus how the chromatin fiber is shaped or, in other words, which mechanisms lead to the emergence of "ordered disorder" inside the cell nucleus.

The ability to fold is an inherent feature of a chromatin fiber. It is determined by electrostatic interactions of nucleosomes [40]. However, the specificity of folding (for example, determination of TAD profiles) is assured by additional mechanisms. First of all, partitioning of the genome into active and repressed segments contributes to the shaping of chromatin fiber, as the nucleosomes of active chromatin are less prone to establish internucleosomal contacts due to the high level of histone acetylation [40, 41]. The second mechanism is based on chromatin interactions with the nuclear structures, particularly with the nuclear lamina and nucleolus (Fig. 3). As revealed by both conventional and single-cell Lamin-DamID [34, 42, 43], at least 50% of the mammalian genome establishes contacts with the lamina at considerable frequency. Lamina- and nucleolus-associated domains (NADs) are represented by largely the same set of genomic regions [44, 45]. Association of a certain LAD/NAD with the lamina/nucleolus is stable during the interphase, but after mitosis LADs and NADs could be reshuffled resulting in transition of a NAD from the nucleolus to the lamina, and LAD transfer to the nucleolus [42]. Apparently, this process is completely asynchronous for different LADs/NADs (or their linked groups) in different cells. Because LADs/NADs constitute a considerable portion of the genome and should be exposed on the surface of chromosome territory, their relocation within the nucleus could influence the overall folding of the harboring chromosome contributing cell-to-cell variability.

The third related mechanism is recruitment of genomic loci to the nuclear functional microcompartments, such as speckles, transcription factories, Cajal bodies, Polycomb-bodies etc. [46] (Fig. 3). For example, erythroid-specific genes form a number of alternate pairwise and tripartite complexes with each other at the transcription factories in mouse erythroblasts [7]. This could markedly influence local as well as long-range chromatin contact profile. Cell-to-cell variability of Polycomb-bodies and transcription factories was not analyzed precisely in the published papers on single-cell Hi-C; nonetheless, we suggest that DNA-containing nuclear bodies and factories could be found in high-coverage single-cell Hi-C data (Fig. 3). Indeed, Stevens et al. [29] show that spatial clustering of Polycomb-regulated regions enriched with H3K27me3 as well as contacts between Klf4-bound regions, and certain interactions within *Nanog* gene regulatory network could be identified by the analysis of single-cell Hi-C data. However, the propensity of any two regions of interest to interact with each other in one particular cell is relatively low and challenges robust identification of genomic regions associated with DNA-containing nuclear bodies and transcription factories in single-cell Hi-C datasets.

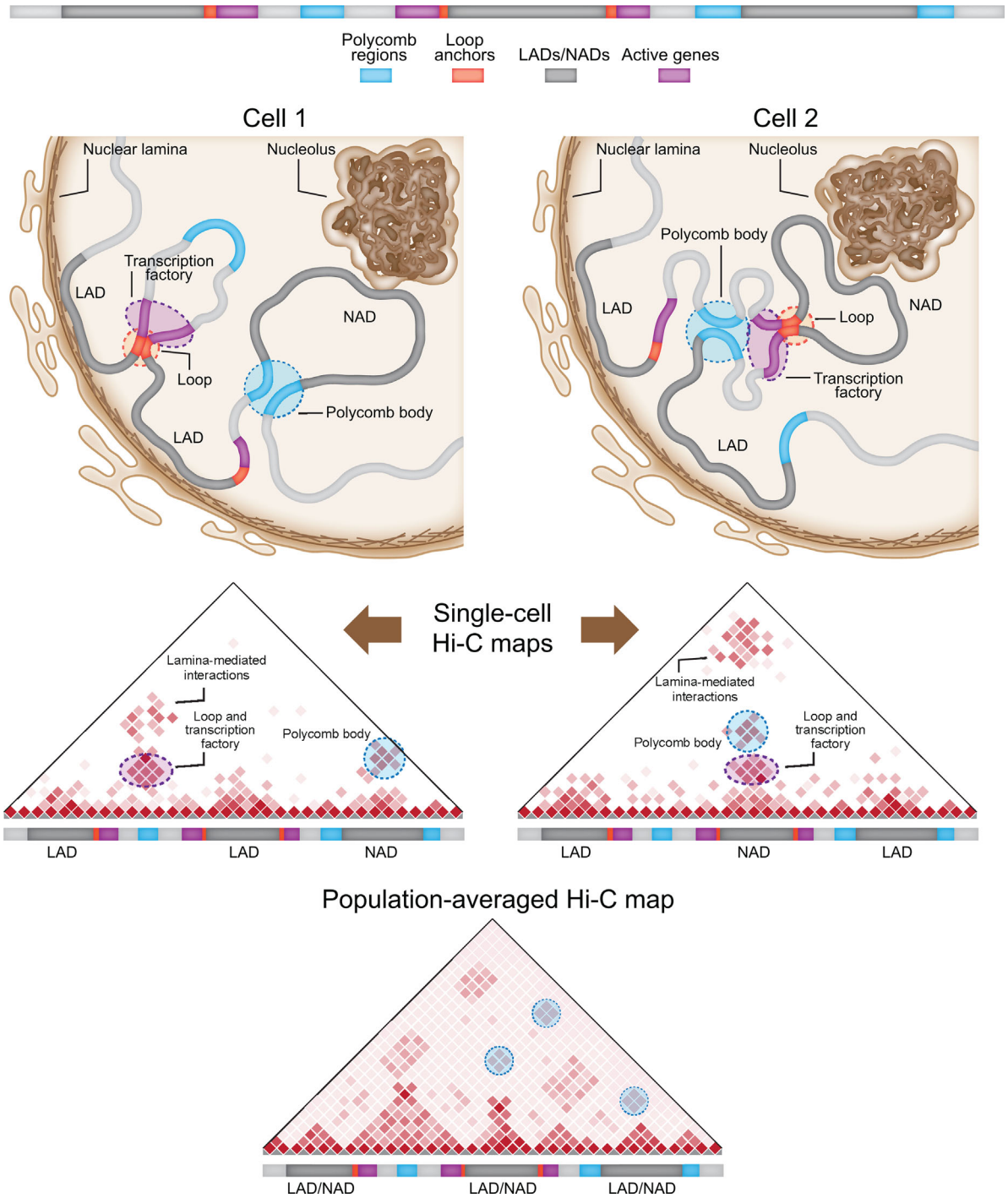


Figure 3. Loops, transcription factories, formation of nuclear bodies and association of chromatin with the nuclear lamina and nucleolus may contribute cell-to-cell variability of the chromatin spatial organization. A schematic representation of a certain genomic region containing several LADs/NADs, active genes, Polycomb-occupied regions and loop anchors is shown at the top of the figure. The central panel shows an illustrative representation of spatial organization of this genomic region in two distinct cells. Hi-C maps of these cells and population-based Hi-C map of the region of interest are shown at the bottom of the figure.

Last but not least, a widely discussed mechanism producing ordered structures from a stochastically folded chromatin fiber is loop extrusion [36, 37, 41, 47–52]. The loop extrusion model postulates that an "extrusion machine" composed of protein motors that are able to move chromatin fibers generates a progressively growing loop, starting at the point where the extrusion machine is loaded onto chromatin until encountering a boundary element. The loop extrusion model proposes an elegant explanation for the genome partitioning into an array of consecutive TAD for interphase and a denser

array of consecutive loops in mitotic chromosomes [36, 47, 49, 53]. Experimental evidence toward this model is mounting and deserves to be discussed in a separate review. Here, we primarily point out the relevance of loop extrusion to the cell-to-cell variability in genome folding. Taking into account the length of chromatin loops that reach up to several megabases [17], one can assume that the extrusion machine should be highly processive; whether this might be promoted by a coordinated action of neighboring extrusion machines at a particular locus remains to be determined. Across the genome, the non-coordinated action of extrusion machines capable of moving millions of base pairs in a relatively short time (~20 minutes [54]) would be expected to largely contribute to a high diversity of chromosome folding within a cell and in a cell population.

To conclude, a variety of molecular mechanisms can shape the intrinsically disordered chromatin fiber producing a diversified pattern of the chromosome spatial structures in individual cells present in the population. The question is whether this diversity is biologically relevant or it simply arises as a result of the stochastic nature of chromatin as a polymer?

Are the dynamics of the chromatin fiber folding functionally relevant?

Capturing the dynamics of chromatin folding will likely require imaging-based methods of live cells. Fixation-based methods such as Hi-C and FISH cannot, in the strictest sense of the word, describe dynamics other than in terms of static changes in chromatin state. The limits of fixation-based methods can be approached by high-resolution time courses [30]. We therefore operate under the assumption that loops and TADs are dynamic structures [36], consistent with their rapid re-establishment in G2 ([55] and bioRxiv 139782).

The dynamics of chromatin fiber folding are presumably important for genome functioning. As a matter of fact, organization and functioning of all biological systems depends critically on their instability. We speculate that the stochasticity of nuclear organization and chromatin folding is generally biologically relevant for at least two reasons. First, stochastic movement of the chromatin fiber allows for selection of functionally significant configurations and thus represents a mechanism of establishing and re-establishing profiles of enhancer-promoter contacts and, consequently, gene expression profiles. Flexibility of the chromatin fiber revealed by single-cell Hi-C experiments is not a bug but a feature that underlines the ability of cells to adapt to a changing environment and react to external stimuli such as hormones, cell-to-cell signaling, and niche environments. Second, stochastic movement of chromatin fiber creates a number of alternative configurations. In the course of cell differentiation, expression of particular sets of transcription factors is likely to allow "freezing" or at least preferential retention of certain configurations favorable for expression of lineage-specific genes. If so, then the testable prediction is that overall variability of chromatin fiber configuration should decrease during the course of differentiation, and increase upon reversion of differentiation (i.e. in iPSCs).

Concluding remarks and future perspectives

Better understanding of the biological consequences of cell-to-cell variability in genome folding will requires a combinatorial approach combining single-cell Hi-C and single-cell RNA-seq from the same cell and comparing single-cell Hi-C with 3D FISH and live-cell imaging of genomic loci. An exciting perspective is to be able to specifically label the newly synthesized RNA to directly capture the consequences of loop extrusion and transcription. At the technical level, improvements in whole genome amplification [56] will protect against sequencing biases and increase the resolution of single-cell data. The combinatorial approaches that will become possible for single-cells will open a new dimension into the inner workings of the nucleus, and may in future necessitate a major revision in our understanding of 3D genome organization.

Acknowledgments

We are grateful to Leonid Mirny, Ilya Flyamer, and Hugo Brandao for discussions and critical reading of the manuscript. S.V.U and S.V.R. were supported by the Russian Science Foundation (project 16-14-10081). K.T.K. is funded by the Austrian Academy of Sciences and the European Research Council (ERC-StG-336460 ChromHeritance).

The authors declare no conflict of interest.

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