TADs or no TADS: lessons from single-cell imaging of chromosome architecture

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Abstract

Eukaryotic genomes are folded in a hierarchical organization that reflects and possibly regulate their function. Genome-wide studies revealed a new level of organization at the kilobase-to-Megabase scale termed “topological associating domains” (TADs). TADs are characterized as stable units of chromosome organization that restrict the action of regulatory sequences within one “functional unit”. Consequently, TADs are expected to appear as physical entities in most cells. Very recent single-cell studies have shown a notable variability in genome architecture at this scale, raising concerns about this model. Furthermore, the direct and simultaneous observation of genome architecture and transcriptional output showed the lack of stable interactions between regulatory sequences in transcribing cells. These findings are consistent with a large body of evidence suggesting that genome organization is highly heterogeneous at different scales. In this review we discuss the main strategies employed to image chromatin organization, present the latest state-of-the-art developments and propose an interpretation reconciling population-based findings with direct single cell chromatin organization observations. All in all, we propose that TADs are made of multiple, low-frequency, low-affinity interactions that increase the probability, but are not deterministic, of regulatory interactions.

Keywords: topologically associating domains, genome architecture, Oligopaint, fluorescent *in situ* hybridization, superresolution microscopy, chromosome conformation capture, promoter-enhancer contacts, chromatin loops.

Highlights

- Eukaryotic genomes are organized in a hierarchical multiscale fashion
- We focus on the contribution of novel imaging technologies to chromatin organization
- There is a high degree of stochasticity in genome folding in single-cells
- TADs are functional units but not necessarily stable physical entities
- The stochastic nature of folding is likely essential to regulate chromatin transactions

Introduction

Storing large amounts of information in small spaces while being able to readily access it rapidly and efficiently remains a current technological challenge of man-made storage devices. Eukaryotic cells face a similar challenge as they need to pack around two meters of linear DNA into a micrometer-sized nucleus. Yet, despite this remarkable degree of compaction, the information contained in the DNA sequence is replicated and translated in a very short amount of time and with great accuracy to ensure inheritance to daughter cells and continuous normal cellular functioning.

At the nucleus, the linear genome is organized in a hierarchical multiscale fashion ranging from a few kilobases to megabases that is, in most cases, reflected by the three-dimensional (3D) folding of chromatin (Fig. 1). Additionally, the minimal structure of chromatin, the nucleosome, represented by DNA wrapped around specific proteins (histones), can carry different covalent chemical modifications that will determine how DNA will interact with transcriptional and reparation machineries and thus chromatin state (for an extended review see (Klemm, Shipony, and Greenleaf 2019)). Compact and repressed chromatin (heterochromatin) domains are usually found at the nucleus periphery and segregated from active and large (eu-)chromatin domains (Frenster, Allfrey, and Mirsky 1963) where chromatin is thought to be actively transcribed (Weintraub and Groudine 1976). On a larger scale, it is now well accepted that individual chromosomes are organized into territories (CT) (Bolzer et al. 2005; Cremer and Cremer 2010) and rarely intermingle with each other. However, the internal organization of chromosomes remained largely unknown until recently. Over the past decade, several breakthroughs in high-throughput sequencing enabled the mapping of pairwise interactions between genetic loci with genomic specificity and genome-wide coverage (Rao et al. 2014; Dekker et al. 2002; Lieberman-Aiden et al. 2009a; de Wit and de Laat 2012) unveiling novel

hierarchical levels of eukaryotic chromatin organization. Topologically associated domains (TADs) have been defined as regions displaying enriched interactions with neighbouring DNA, and appear in Hi-C matrices as squares on the main diagonal (Dixon et al. 2012; Nora et al. 2012; Sexton et al. 2012). Thus, the definition of TADs describes a novel feature of Hi-C maps, and does not involve any specific factor (e.g. cohesin) or mechanism (e.g. loop extrusion) (Alipour and Marko 2012). Finally, active/repressed TADs often associate with each other in the nucleus to form active (A) or inactive (B) compartments (Fig. 1) (Sexton et al. 2012; Dixon et al. 2012; Nora et al. 2012; Lieberman-Aiden et al. 2009b; Schmitt, Hu, and Ren 2016; Rao et al. 2014).

Hierarchical organization critically impacts nuclear activities such as transcription, replication as well as cellular events such as cell cycle regulation, key cell fate decisions and embryonic development, yet the mechanisms and the exact role of each structural element, particularly at the TAD level, is still a matter of debate. Particularly, from genome wide methods two contrasting hypothesis regarding the role of TADs and their structural interpretation has arisen (see next section). Fluorescent microscopy is in a privileged place to reveal the nuclear organization in single cells and settle this apparent contradiction. The aim of this review is to present an overview of microscopy findings over the last decade that lay the bases of present discoveries and the current state-of-the-art on high-throughput-high-content imaging technologies. We will put into context and discuss the results from these findings to present a general picture of genome organization and a new interpretation that reconciles both models.

Are TADs a stable structural unit of chromatin organization or a statistical phenomena?

TADs range in size from tens of kilobases to megabases and are defined as self-interacting genomic regions: genomic loci within a TAD display a higher probability of

interacting with loci belonging to the same TAD than with other genomic locations. TADs display remarkable correlations with coordinated gene expression (Dixon et al. 2012; Le Dily et al. 2014), epigenetic histone modifications (Dixon et al. 2012; Sexton et al. 2012; Hou et al. 2012), and with replication timing (Pope et al. 2014; Dixon et al. 2012). In addition, TADs exhibit a surprising developmental and evolutionary robustness (Nora et al. 2012; Dixon et al. 2015; Vietri Rudan et al. 2015). The current paradigm posits that TADs restrict the action of most regulatory sequences, such as enhancers, within one "functional unit" (Shen et al. 2012; Symmons et al. 2014; Ghavi-Helm et al. 2014; Neems et al. 2016; Ji et al. 2016; Dowen et al. 2014; Ron et al. 2017; Lupiáñez et al. 2015). Furthermore, TAD boundaries were proposed to form stable loops to achieve insulation between neighbouring domains (Hug et al. 2017; Rao et al. 2014). Taken together, these data suggest that TADs play a pivotal role in the organization of chromosomes and that they represent stable structural units of chromosome organization. A clear expectation from this model is that TADs should exist as physical entities in most cells.

Contrasting this hypothesis, single-cell genome-wide studies have revealed a notable variability in chromosome spatial architecture both at the intra- and inter-TAD levels (Nagano et al. 2017; Stevens et al. 2017; Flyamer et al. 2017; Kind et al. 2015). Clusters of contacts in single cells did not necessarily match TADs as predicted from ensemble Hi-C, indicating that often loci within TADs contact loci in neighboring TADs (Flyamer et al. 2017).

These findings were consistent with a large body of evidence showing —even before the existence of TADs was revealed— that chromosome organization is highly heterogeneous at the single cell level (Lanctôt et al. 2007). More recently, Fluorescence In Situ Hybridization (FISH) and polymer simulations have shown extensive variability in genome architecture at the level of TADs (Nora et al. 2012; Giorgetti et al. 2014; Ulianov et al. 2016). Overall, these data suggest

that TADs may arise due to ensemble averaging effects, and raises the question of how such a variable TAD structure may be relevant for transcriptional regulation.

What imaging technologies have revealed so far about chromatin organization

The imaging strategies to study genome organization can be, according to the labeling approach, divided mainly in two: 1) the labeling of a nuclear protein associated to chromatin or 2) the labeling of specific DNA/RNA sequences. The first approach relies on inmuno recognition, usually of the endogenous protein, or marking the protein of interest with a fluorescent tag, which usually involves ectopic expression. This results in a labeling at the level of the whole nucleus. Particularly interesting is the combination of this labeling approach with super-resolution microscopy in recent times. Single Molecule Localization microscopy (SMLM) consists of isolating single fluorophore emitters stochastically activated by repeated imaging and fitting of the resulting signal to reconstruct a pointilistc image, down to a lateral resolution of ~20 nm (Betzig et al. 2006; Hess, Girirajan, and Mason 2006; Rust, Bates, and Zhuang 2006). When labeling histone H2B, structural component of nucleosomes, through immunochemistry in fixed cells employing Stochastic Optical Reconstruction Microscopy (STORM), Ricci et al. found discrete nanosized chromatin domains in mammalian cells (Ricci et al. 2015). The chromatin domain sizes correlated well with cell pluripotency, with stem cells displaying smaller domains. More recent work employed photoactivated localization microscopy (PALM) to image a H2B-mCherry fusion protein in live cells (Nozaki et al. 2017). Groups of nucleosomes move coherently, forming clustered domains. The average size of the observed domains was much higher than previously found, with a peak diameter of ~160 nm. Second, labeling histone marks using STORM revealed nanosized nucleosome “nanoclusters” or “nanodomains” in both fly and mammals (Fig. 2a) (Cattoni et al. 2017; Xu et al. 2018). Notaly, repressive domains were

considerably larger than active domains (Cattoni et al. 2017; Xu et al. 2018), consistent with predictions from genome-wide data (Filion et al. 2010). In addition, domains types displayed very low co-localization, suggesting the orthogonality of active and repressive marks at the single cell level. Finally, imaging Polycomb group proteins (by labeling Polycomb-repressed domains) with STORM showed that the nanoscale organization of TADs facilitates long-range chromatin interactions and have direct consequences to gene expression (Wani et al. 2016). As was previously suggested (Bantignies et al. 2011), clustering of Polycomb domains is involved in gene regulation, providing a link between chromatin topology and transcription.

A main limitation of immunolabeling of histone marks coupled to super-resolution is the lack of genomic specificity. Recently, a number of technologies have emerged that are able to circumvent this limitation. FISH is a widely used technique to label specific genomic regions in chemically fixed cells by the hybridization of fluorescently-labeled DNA probes to a complementary genomic region of interest (Rudkin and Stollar 1977; Bauman et al. 1980). 3D imaging of FISH-labeled genomic regions allows for the quantitative measurement of distances between different genomic loci, or the distance of a specific locus to a given nuclear structure (see (Foster and Bridger 2005; Huber, von Voithenberg, and Kaigala 2018) for a review). For instance, FISH enabled the measurement of the relocalization of an endogenous gene locus from the nuclear periphery towards the nuclear interior upon transcriptional activation in mammalian cells (Williams et al. 2006; Zink et al. 2004), suggesting a mechanism of gene regulation that relies on the positioning of genes at specific distances from the nuclear lamina (reviewed in (Shachar and Misteli 2017)).

In the past, FISH probes were generated through cloning in bacterial artificial chromosomes (BACs) or fosmids. Preparation of these probes is time-consuming and produces relatively large genomic probes (50-100 kb). Oligonucleotide-based FISH, or Oligopaint, is a

novel technology that allows for bioinformatic design and direct labeling of genomic regions of variable length, from a few kilobases to Megabases (Mb) (Beliveau et al. 2012, 2015). A pioneering study by Boettiger *et al.* combined oligopaints with super-resolution microscopy (STORM) to image epigenomic domains in *Drosophila* cultured cells (Boettiger et al. 2016). This study reported three different packaging ratios for epigenetically distinct genomic regions. Notably, Polycomb-repressed domains showed a compact packing and less than 3% overlap with neighbouring active domains whereas inactive domains displayed up to a 15% overlap with neighbouring active domains. Employing a similar approach, but labeling contiguous TADs, Szabo et al. directly observed “nano-compartments” that corresponded to repressed domains interspersed with active chromatin domains (Fig. 2b) (Szabo et al. 2018). Overall, these results were in agreement with previous polymer simulations (Ulianov et al. 2016).

Imaging of entire domains provides valuable single-cell information on the degree of cell-to-cell structural variation of chromatin domains, but still lacks in genomic specificity to fully assess the relevance of this heterogeneity. For instance, this technique does not allow the probing of the absolute frequency of interactions of two loci within the same TAD or between neighbouring TADs. To address this issue, we combined Oligopaint and super-resolution microscopy 3D Structured Illumination Microscopy (3D-SIM) to image and label genomic loci spanning consecutive TADs borders or within single *Drosophila* TADs (Cattoni et al. 2017). We found a great cell-to-cell variability in the observed distances between TAD borders. Contact frequencies can increase many-fold without large changes in the average or median distance (Giorgetti and Heard 2016), thus we turned to the calculation of the absolute contact probability, which can be estimated by integrating the distance probability density between zero and the colocalization precision limit (~120 nm). The absolute frequency of contacts between or within TADs was relatively small (<10% in all cases). Notably, contacts between pairs of loci within

TADs were only about 2-3 fold higher that contacts between neighbouring TADs, suggesting that the folding of chromatin into “separate” TADs may require only small differences in absolute contact probabilities and that violations of insulation between TADs may occur rather frequently.

Simultaneous imaging of tens of genomic regions in single cells at high resolution

The combination of FISH and super-resolution microscopies shed light into the structural heterogeneity of chromatin folding into TADs and on the relative condensation levels of different TAD types. But, further structural insight requires the ability to visualize multiple genomic regions in the same cell at once. Early work introduced color barcoding in FISH to reveal the position of 13 genomic loci spaced across an entire *Drosophila* chromosome arm using spatial constraints (Lowenstein, Goddard, and Sedat 2004). More recently, a multiplexed strategy based on sequential labeling was developed for single molecule RNA-FISH (Lubeck et al. 2014; K. H. Chen et al. 2015). These studies introduced a new concept for multiplexed imaging relying on sequential cycles of probe hybridization, imaging, and probe removal to access the number and position of tens of transcripts. Using a similar concept, Wang et al. employed the versatility of Oligopaint coupled to sequential imaging to directly label the central regions (100 kb) of 32 TADs spanning an entire chromosome of human IMR90 cells (Wang et al. 2016). This study revealed that TADs of the same epigenetic type tend to associate spatially in single cells and that this association is highly variable between cells. A more recent work used STORM to image 9 consecutive TADs in a human chromosome and reached similar conclusions (Nir et al. 2018).

More recently, three studies labeled dozens of single genomic loci (~2-30 kb in size) and visualized them using sequential imaging to reveal chromatin organization at the TAD scale (Cardozo Gizzi et al. 2019; Bintu et al. 2018; Mateo et al. 2019). Bintu *et al.* imaged consecutive 30-kb segments covering 1.2 to 2.5 Mb in human cultured cells (Bintu et al. 2018) while Mateo

et al. (Mateo et al. 2019) and ourselves (Cardozo Gizzi et al. 2019) focused on the visualization of chromatin folding at shorter genomic scales (2-10kb) in *Drosophila* embryos. Strikingly, the average contact maps obtained from microscopy data exhibited a very strong correlation with the TAD organization observed by Hi-C at all scales analyzed (Fig. 3a) (Mateo et al. 2019; Bintu et al. 2018; Cardozo Gizzi et al. 2019). Using STORM, Bintu *et al.* observed domain structures with globular conformations and sharp boundaries in single human cells (Bintu et al. 2018), compatible with the previously described “nanodomains” (Szabo et al. 2018). Notably, the boundaries of these nanodomains varied from cell to cell. A remarkable finding of this study is that the probability of finding a domain boundary in a single cell was highest at the location of TAD borders annotated by Hi-C, but the absolute contact probabilities remained small (~10-25%), consistent with our previous findings (Cattoni et al. 2017).

Several modeling approaches also explored the question of variability in chromosome organization at the TAD level. Copolymer models describe chromatin as chain of monomers of different epigenetic states, with monomers of the same epigenetic type displaying preferential interactions (Jost et al. 2014; Kim et al. 2016). These models have suggested that TADs arise from the internal collapse of epigenomic domains and that TADs are actually close but above the theta-collapse transition, i.e. in a phase where fluctuations of TAD structure are large and clustering weak and dynamic (Jost et al. 2014; Lesage et al. 2019).

The heterogeneity of TAD structures in mammals is also consistent with the loop extrusion model (Alipour and Marko 2012; Fudenberg et al. 2016; Sanborn et al. 2015). In this model, the binding of cohesin complexes to chromatin creates a loop. The translocation of cohesin rings from their anchoring point leads to the formation of progressively larger (‘extruding’) loops. This process continues until one or both cohesin anchors dissociate from chromatin, or until cohesin encounters a CTCF-bound site (typically located at TAD borders).

This model is thus consistent with a large heterogeneity in TAD structures (Oudelaar et al. 2018). Therefore, both copolymer and loop extrusion polymer models both predict large intrinsic variability in the spatial organization of TADs.

In agreement with the loop extrusion model, cohesin depletion caused the disappearance of TADs at the ensemble level (Rao et al. 2017; Schwarzer et al. 2017; Bintu et al. 2018). Interestingly, at the single-cell level, cohesin did not seem to be required to maintain domain structures but rather to establish preferred TAD borders (Bintu et al. 2018). In our view, more studies will be required to determine whether the domains observed by single-cell imaging methods in absence of cohesin do not merely reflect the polymer nature of the chromatin fiber.

A different model for chromatin organization that is compatible with these findings is the liquid-liquid phase separation (LLPS) (Hnisz et al. 2017; Maeshima et al. 2016). Such a model requires the existence of multivalent interactions between chromatin-associated proteins to form self-aggregating condensates. The existence of a phase transition mediated by protein factors with affinity to either active or inactive chromatin (Strom et al. 2017; Larson et al. 2017; Cho et al. 2018; Boehning et al. 2018) would also support a possible mechanism of compartmentalization.

A strict LLPS model would be, however, difficult to reconcile with single-cell measurements of absolute contact frequencies within and between TADs in *Drosophila* and in mammals (Cardozo Gizzi et al. 2019; Finn et al. 2019). These studies showed that absolute contact frequencies within TADs were relatively small (2-9% depending on epigenetic context), while loci located across TAD borders displayed comparable contact frequencies (~3%) (Cardozo Gizzi et al. 2019; Finn et al. 2019). Similar findings were reported by single-cell Hi-C (Flyamer et al. 2017) or by single-cell measurements of the overlap frequencies of neighbouring TADs (Boettiger et al. 2016; Szabo et al. 2018). Overall, these results would indicate that

neighboring TADs would need to have a considerable degree of miscibility, in contrast to traditional LLPS models.

A second expectation from canonical LLPS models would be that phase-separated binding factors should appear as spherical objects. If one assumes that single TADs occupy the volumes defined by phase-separated DNA binding factors, then one would expect to observe spherical TADs in most cells. Single-cell visualization of TADs, however, showed that TAD shapes are most often not spherical (Boettiger et al. 2016; Szabo et al. 2018; Oudjedi et al. 2016). However, direct, single-cell, super-resolved visualization of putative phase-separated factors would be needed to directly establish whether these proteins can form spherical objects. Finally, canonical LLPS models would predict that TADs of the same epigenetic outlook (e.g. Polycomb) would fuse into single droplets when they encounter each other. However, contacts between repressive TADs are rare and display short-lived dynamics (Cattoni et al. 2017; Cheutin and Cavalli 2012; Bantignies et al. 2011). Thus, a more sophisticated LLPS model accounting for the underlying properties of the chromatin matrix may be required to account for these observations (Erdel and Rippe 2018).

Internal contacts within TADs happen at low-frequencies, but any given locus is potentially capable of contacting a myriad of other loci within its TAD. Thus, the combined probability that a locus contacts another locus within the same TAD is actually relatively high: 70% (Cardozo Gizzi et al. 2019). This suggests that TADs are made of multiple, low-frequency, low-affinity interactions that would be sufficient to hold them together to form discrete, though highly variable structures. In this interpretation, TAD folding would be driven by a combination of three types of interactions: (1) a meshwork of intra-TAD contacts involving regulatory and genetic elements (e.g. enhancers, promoters, insulators), (2) contacts mediated by cohesin/condensin or other factors (acting by loop-extrusion or other mechanisms), and (3)

changes in chromatin structure induced by other DNA management processes (e.g. transcription, replication, repair). These three mechanisms are dynamic and therefore their combined, often uncorrelated action would independently contribute to a large degree of cell-to-cell heterogeneity in the organization of TADs. Therefore, it becomes essential to be able to measure chromosome organization and the status of other DNA management processes (e.g. replication, transcription) simultaneously in single cells.

Chromatin architecture and transcriptional status in single cells: re-interpreting the link between chromatin folding and gene expression

An early study reported that single-cell RNA expression and TAD compaction were correlated (Giorgetti et al. 2014). Interestingly, volumes of TADs within inactive and active X-chromosomes where anti-correlated. In fact, expression of one of the genes within this TAD (*Tsix*) tended to be higher in the allele with the smaller TAD, whereas expression of a second gene within the same TAD (*Linx*) correlated instead with larger TAD volumes. This indicates that gene expression can impact in different manners the overall shape of a TAD.

Recently, two studies combined RNA detection with DNA sequential imaging schemes to simultaneously reconstruct chromatin architecture and transcriptional status in single cells (Cardozo Gizzi et al. 2019; Mateo et al. 2019). Cardozo Gizzi *et al.* reconstructed the changes in chromatin structure during development of a TAD containing *snail*, a key developmental activator exhibiting a well-defined and well-characterized spatio-temporal expression pattern (Fig. 3b). Hi-M was performed at different developmental times on intact *Drosophila* embryos, and nascent *snail* transcripts were recorded by tyramide amplification, a methodology that permits simultaneous RNA- and DNA-FISH detection without sample unmounting and re-hybridization. We observed that TADs emerge primarily between nuclear cycles 13-14,

consistent with previous findings by Hi-C (Hug et al. 2017). Strikingly, chromatin within the *snail* TAD was locally decondensed in nuclei displaying *snail* expression. This local decondensation lead to an internal reconfiguration of the TAD structure, consistent with genome architecture being affected by, or being prepared for, transcriptional activation during embryo development (Cardozo Gizzi et al. 2019). These results are consistent with local Polycomb-mediated decondensation occurring before transcriptional activation (Cheutin and Cavalli 2018).

Mateo et al. developed a conceptually similar methodology to simultaneously visualize RNA and DNA in single-cells (Mateo et al. 2019). In this approach, cryo-sections of *Drosophila* embryos were first hybridized with RNA probes and sequentially imaged to obtain the localization patterns of 30 RNA species. Next, the same cryo-section was re-hybridized with DNA probes and imaged sequentially to obtain the positions of up to 55 genomic loci. This study specifically focused on the *Bithorax* complex, responsible for determining the posterior thorax and abdominal segments in the fly. Interestingly, the correlation between enhancer-promoter (E-P) spatial proximity and transcriptional state was rather weak, suggesting that enhancers do not remain in close contact to promoters during transcriptional elongation. Further, Chen at al. performed live microscopy in *Drosophila* embryos to reveal that direct contacts between *eve* enhancers and the promoter of a reporter gene are necessary but not sufficient for continuous initiation of transcription (H. Chen et al. 2018). Overall, these studies are consistent with enhancer-promoter contacts being required to initiate transcription but not for elongation. Many genes are transcribed by episodic bursts of RNA synthesis (Raj and van Oudenaarden 2008; Paulsson 2004; Rodriguez et al. 2019). Taking these results together, it is perhaps not surprising that E-P contacts are not consistently observed in nuclei displaying active transcriptional hotspots.

Intriguingly, a very recent study has challenged this notion by showing that transcriptional activation of *Sox2* in live mouse embryonic stem cells (mESCs) is not correlated with the spatial proximity between the *Sox2* promoter and its essential enhancer (Alexander et al. 2019). In a second study, it was shown that upon differentiation of mESCs to neural precursors, the *Shh* gene is expressed but the E-P spatial distance is *increased (Benabdallah et al. 2019)*. These studies suggest a complex relationship between genome topology, transcription and differentiation. All in all, these results indicate that 3D chromosome organization likely plays a key role in the regulation of transcriptional activation and enhancer function, but that the mechanisms involved require further investigation.

Conclusions and future perspectives

Nuclear architecture is much more heterogeneous than originally anticipated from genome-wide biochemical methods. A wealth of data from imaging and single-cell methods are therefore inconsistent with the existence of stable, deterministic 3D folding structures. In information science, noise is generally the enemy of information, however a tolerable amount of noise is a source of freedom and warrants flexibility (Barkai and Shilo 2007; Patange, Girvan, and Larson 2018). For instance, the ability of chromatin to explore a wide range of conformations enables promoters to be contacted by different enhancers in different tissues or developmental stages. On the other hand, this large conformational flexibility of the genome means that other layers of information, such as epigenetics or transcription factor occupancy, are also necessary to achieve proper gene regulation.

Despite the important recent advances in imaging technologies, several key aspects remain to be improved to gain further insight into chromosome organization and function. First, current live imaging technologies allow for the tracking of a limited number of transcripts/ DNA

loci at any given time (at most three) (H. Chen et al. 2018). The extension of these technologies to enable live, multiplexed detection of more species would uniquely permit the dissection of how chromatin is pre-/re-organized before and during transcriptional activation, replication origin licensing, recombination, or repair. An increase in the number of species visualized should allow for the detection of the dynamics of TAD folding to ultimately link TAD structural dynamics to transcriptional kinetics.

Second, multiplexed detection of RNA and DNA has been recently achieved and used to study the links between chromatin folding and transcription (Mateo et al. 2019; Cardozo Gizzi et al. 2019). The multiplexed combination of FISH and immunolabeling would further enable the detection of what epigenetic outlook or protein factors are responsible for specific DNA folding patterns and transcriptional outputs. Third, current super-resolution microscopies have been used to reveal chromosome organization at the 30-100 nm scales (Ricci et al. 2015; Cattoni et al. 2017; Boettiger et al. 2016; Szabo et al. 2018). To gain further insight, it will be necessary to: (1) improve probes to provide multiplexing abilities to live super-resolution microscopies, (2) rely on new technologies with higher spatial resolutions, such as MINFLUX (Balzarotti et al. 2017) or electron microscopy (Ou et al. 2017); and (3) develop new sample fixation methods adapted for sub 10-nm-scale imaging. These new developments in optical imaging technologies will likely continue to revolutionize our understanding of chromosome organization and function in the near and far future.

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Author Contributions

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest

The authors declare no competing interests.

Figure 1

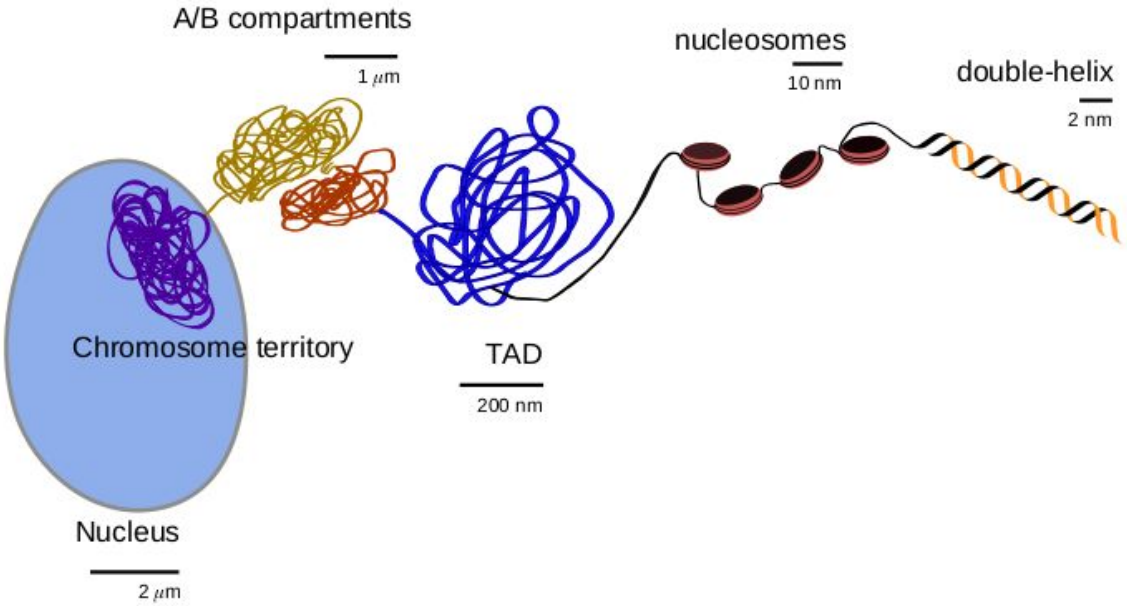

Figure 1. Simplified scheme of the multiscale hierarchical organization of chromatin in eukaryotic organisms. The DNA double helix folds into the ~11-nm fiber chromatin fiber by wrapping 147 base pairs of DNA around a histone octamer. At the intermediate scale, chromatin is organized in TADs, domains of preferential contacts. The association of TADs of the same epigenetic type give rise to A/B compartments. Active chromatin (A) tends to interact with other active segments, whereas the same is true for repressive chromatin (B). At the level of the whole chromosome, individual chromosomes occupy its own area in the nucleus, forming chromosomal territories.

Figure 2

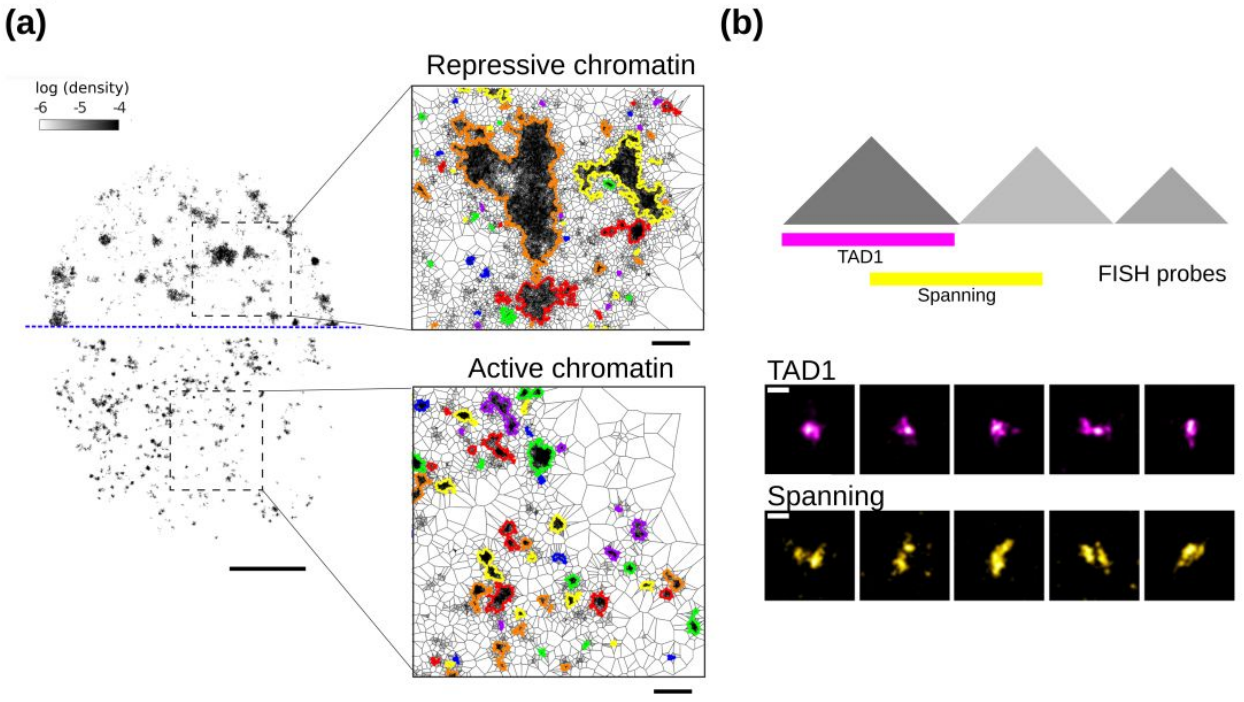

Figure 2. (a) dSTORM images of Alexa-647-labelled epigenetic marks H3K27me3 (repressed chromatin) and H3K4me3 (active chromatin). Images show density maps computed from the area of polygons obtained from the Voronoï diagram with scale defined on top. Scale bar = 1 µm. Zoomed regions display detected nanodomains (highlighted with different colors). Scale bar = 200 nm. Image has been adapted from (Cattoni et al. 2017). **(b)** Upper panel, scheme of FISH probe position. TAD1 probe (magenta) covers the whole TAD while Spanning probe (yellow) has the same genomic size as TAD 1, 198 kb, but shifted to span the boundary. Lower panel, representative examples of dSTORM imaging of TAD 1 (magenta) and Spanning (yellow) probes. While TAD1 displayed only one nano-compartment in the majority of cells, Spanning probe split into two or more nano-compartments. Scale bars: 250 nm. Image has been adapted from (Szabo et al. 2018).

Figure 3

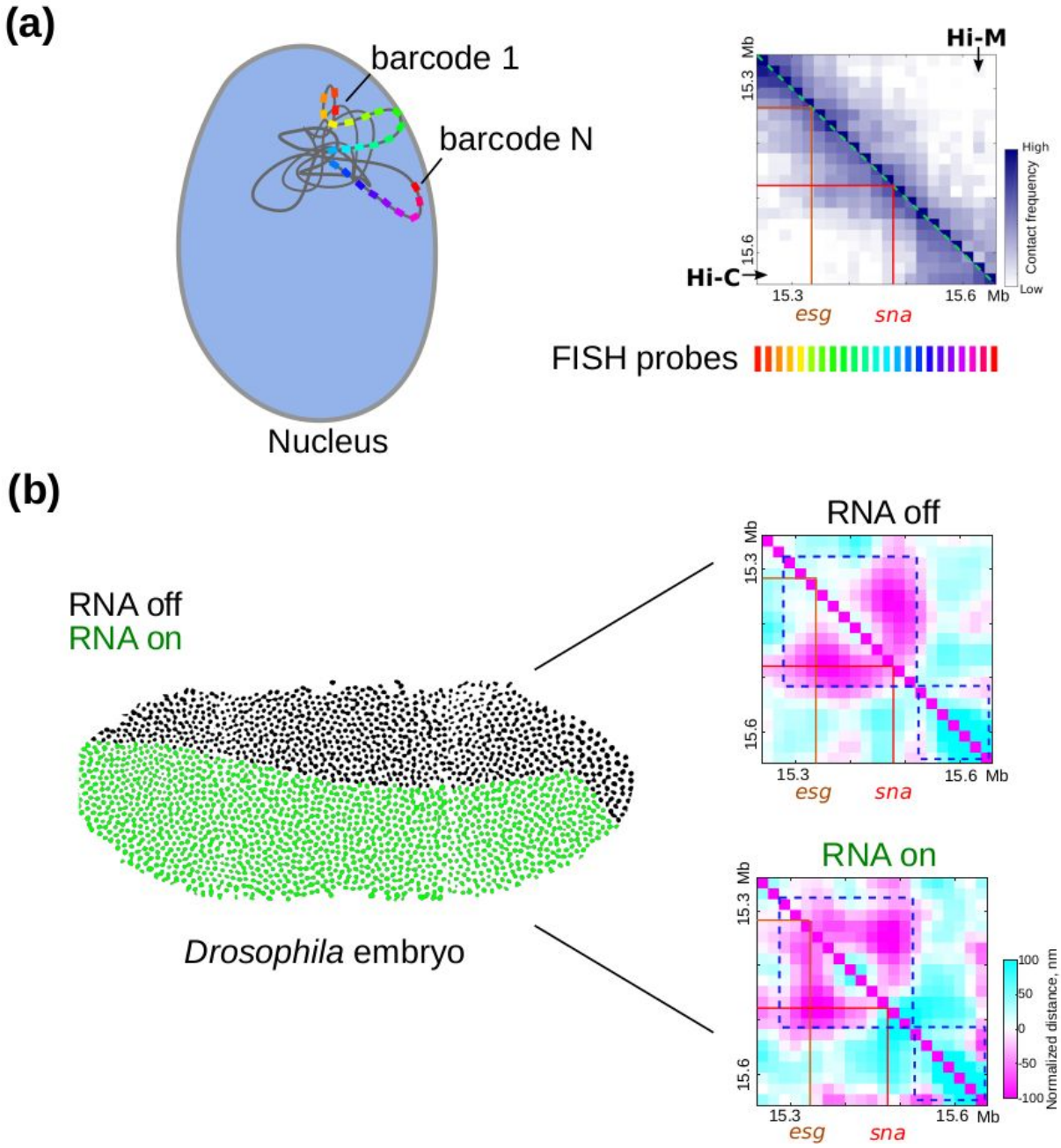

Figure 3. (a) Left, schematic representation of a section of the chromatin fiber in a blue-stained nuclei. Barcode 1 to N are depicted as rectangles with a color code following their genomic location. Right, Hi-M map and interpolated Hi-C matrix from nc 14 wild-type embryos spanning the 400-kb region encompassing *sna* and *esg*. Relative (Hi-C) and absolute (Hi-M) contact

frequencies are color-coded according to the scale bar. **(b)** Left, full segmented *Drosophila* embryo. Nuclei expressing *snail* are shown in green, whereas nuclei not expressing *snail* appear in black. Right, normalized Hi-M mean physical distance maps for nuclei expressing (bottom) or not expressing (top) *snail*. The color scale indicates distances shorter (magenta) or higher (cyan) than expected (scale in nanometers). Solid lines represent the positions of *snail* (sna) and *escargot* (esg). TADs locations are delineated with dotted blue lines. Chromatin in the vicinity of *snail* was locally decondensed in transcribing nuclei, indicated by the mean distances higher than expected. Panels **(a)** and **(b)** have been adapted from (Cardozo Gizzi et al. 2019).

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