# 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 regulates their function. Genomewide 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.



## 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 2 m 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 Ref. [1]). Compact and repressed chromatin (heterochromatin) domains are usually found at the nucleus periphery and segregated from active and large (eu-)chromatin domains [2] where chromatin is thought to be actively transcribed [3]. On a larger scale, it is now well accepted that individual chromosomes are organized into territories (CT) [4,5] 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-



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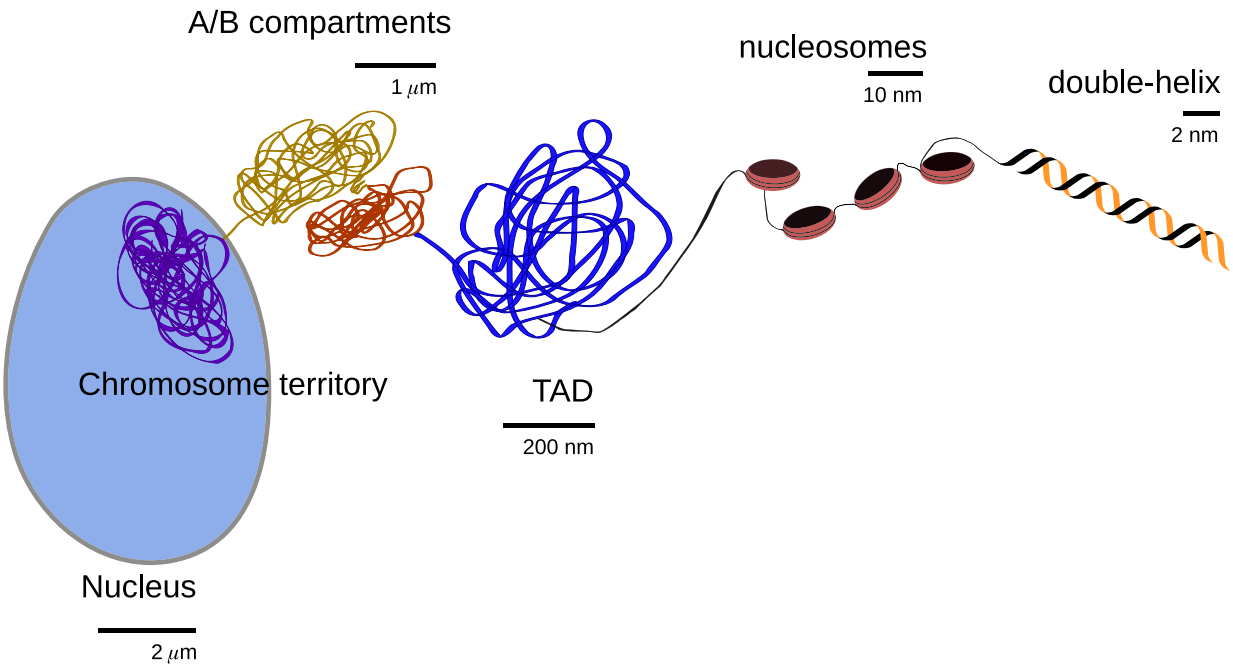


**Fig. 1.** Simplified scheme of the multiscale hierarchical organization of chromatin in eukaryotic organisms. The DNA double helix folds into the ~11-nm chromatin fiber by wrapping 147 base pairs of DNA around a histone octamer. At the intermediate scale, chromatin is organized in TADs, domains of preferential contacts. The association of TADs of the same epigenetic type gives 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 their own area in the nucleus, forming chromosomal territories.

throughput sequencing enabled the mapping of pairwise interactions between genetic loci with genomic specificity and genomewide coverage [6–9] unveiling novel hierarchical levels of eukaryotic chromatin organization. Topologically associated domains (TADs) have been defined as regions displaying enriched interactions with neighboring DNA and appear in Hi-C matrices as squares on the main diagonal [10–12]. 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) [13]. Finally, active/repressed TADs often associate with each other in the nucleus to form active (A) or inactive (B) compartments (Fig. 1) [6,10–12,14,15].

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, are still a matter of debate. Particularly, from genomewide methods, two contrasting hypotheses regarding the role of TADs and their structural interpretation have 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 phenomenon?

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 [10,16], epigenetic histone modifications [10,12,17], and replication timing [10,18]. In addition, TADs exhibit a surprising developmental and evolutionary robustness [11,19,20]. The current paradigm posits that TADs restrict the action of most regulatory sequences, such as enhancers, within one "functional unit" [21–28]. Furthermore, TAD boundaries were proposed to form stable loops to achieve insulation

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between neighboring domains [6,29]. 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 [30–33]. 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 [32].

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 [34]. More recently, fluorescence in situ hybridization (FISH) and polymer simulations have shown extensive variability in genome architecture at the level of TADs [11,35,36]. Overall, these data suggest that TADs may arise due to ensemble averaging effects and raise 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 inmunorecognition, 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 pointillistic image, down to a lateral resolution of ~20 nm [37–39]. 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 [40]. 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 [41]. Groups of nucleosomes move coherently, forming clustered domains. The average size of the observed domains was much higher than that previously found, with a peak diameter of ~160 nm. Second, labeling histone marks using STORM revealed nanosized nucleosome "nanoclusters" or "nanodomains" in both flies and mammals (Fig. 2a) [42,44]. Notably, repressive domains were considerably larger than active domains [42,44], consistent with predictions from genomewide data [45]. In addition, domain types displayed very low colocalization, 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 has direct consequences to gene expression [46]. As was previously suggested [47], 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 [48,49]. 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 Refs. [50,51] for a review). For instance, FISH enabled the measurement of the relocalization of an endogenous gene locus from the nuclear periphery toward the nuclear interior upon transcriptional activation in mammalian cells [52,53], suggesting a mechanism of gene regulation that relies on the positioning of genes at specific distances from the nuclear lamina (reviewed in Ref. [54]).

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) [55,56]. A pioneering study by Boettiger et al. combined Oligopaints with super-resolution microscopy (STORM) to image epigenomic domains in *Drosophila* cultured cells [57]. This study reported three different packaging ratios for epigenetically distinct genomic regions. Notably, Polycomb-repressed domains showed a compact

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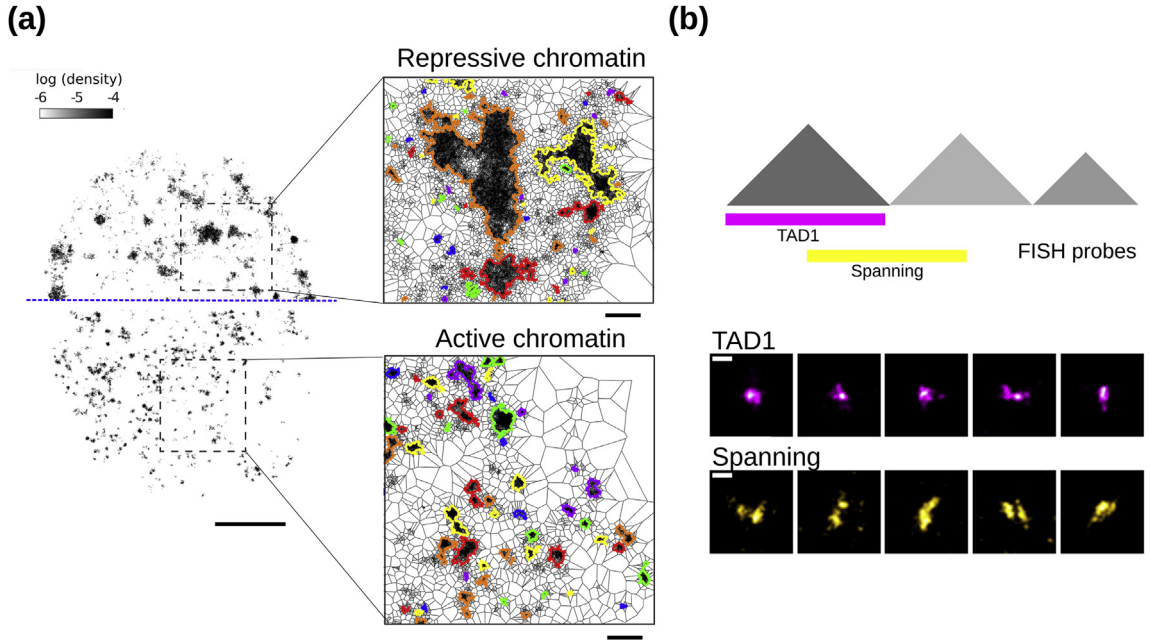


**Fig. 2.** **(a)** dSTORM images of Alexa-647-labeled epigenetic marks H3K27me3 (repressed chromatin) and H3K4me3 (active chromatin). Images show density maps computed from the area of polygons obtained from the Voronoï diagram with scale defined on top. Scale bar = 1 μm. Zoomed regions display detected nanodomains (highlighted with different colors). Scale bar = 200 nm. Image has been adapted from Ref. [42]. **(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 nanocompartment in the majority of cells, spanning probe split into two or more nanocompartments. Scale bars: 250 nm. Image has been adapted from Ref. [43].

packing and less than 3% overlap with neighboring active domains, whereas inactive domains displayed up to a 15% overlap with neighboring active domains. Employing a similar approach, but labeling contiguous TADs, Szabo et al. directly observed "nanocompartments" that corresponded to repressed domains interspersed with active chromatin domains (Fig. 2b) [43]. Overall, these results were in agreement with previous polymer simulations [36].

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 neighboring 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 [42]. We found a great cell-to-cell variability in the observed distances between TAD borders. Contact frequencies can increase manyfold without large changes in the average or median distance [58], 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 than contacts between neighboring 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

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across an entire *Drosophila* chromosome arm using spatial constraints [59]. More recently, a multiplexed strategy based on sequential labeling was developed for single-molecule RNA-FISH [60]; K. H [61]. 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 [62]. 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 nine consecutive TADs in a human chromosome and reached similar conclusions [63].

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 [64−66]. Bintu et al. imaged consecutive 30-kb segments covering 1.2−2.5 Mb in human cultured cells [65] while Mateo et al. [66] and we [64] focused on the visualization of chromatin folding at shorter genomic scales (2−10 kb) 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) [64−66]. Using STORM, Bintu et al. observed domain structures with globular conformations and sharp boundaries in single human cells [65], compatible with the previously described "nanodomains" [43]. 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 [42].

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 [67,68]. 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, that is, in a phase where fluctuations of TAD structure are large and clustering weak and dynamic [67,69].

The heterogeneity of TAD structures in mammals is also consistent with the loop extrusion model [13,70,71]. 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 [72]. Therefore, both copolymer and loop extrusion polymer models 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 [65,73,74]. Interestingly, at the single-cell level, cohesin did not seem to be required to maintain domain structures but rather to establish preferred TAD borders [65]. In our view, more studies will be required to determine whether the domains observed by single-cell imaging methods in the 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) [75,76]. 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 [77−80] 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 [64,81]. 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%) [64,81]. Similar findings were reported by single-cell Hi-C [32] or by single-cell measurements of the overlap frequencies of neighboring TADs [43,57]. 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 [43,57,82]. 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

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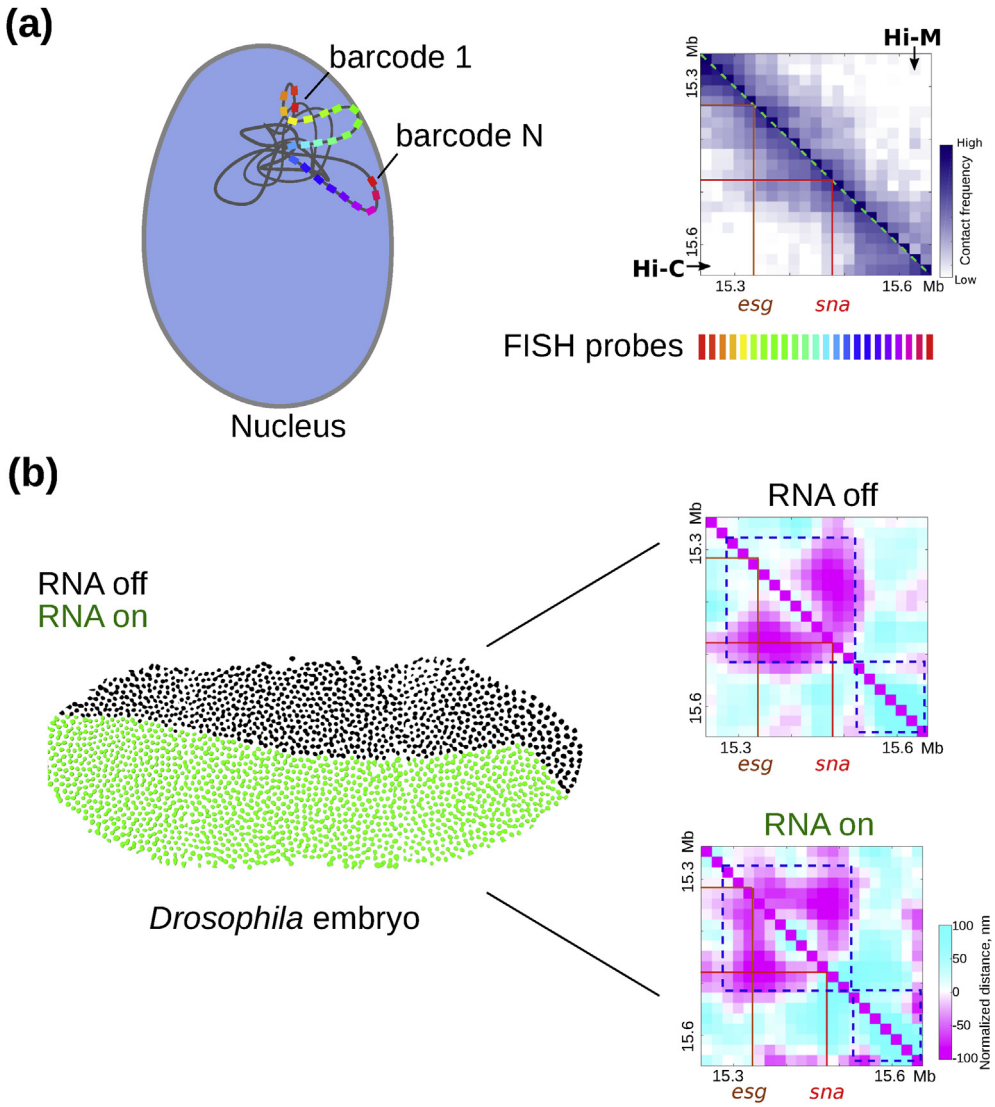


**Fig. 3.** **(a)** Left, schematic representation of a section of the chromatin fiber in blue-stained nuclei. Barcode 1−N are depicted as rectangles with a color code following their genomic location. Right, Hi-M map and interpolated Hi-C matrix from nc 14 wild-type embryos spanning the 400-kb region 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 Ref. [64].

encounter each other. However, contacts between repressive TADs are rare and display short-lived dynamics [42,47,83]. Thus, a more sophisticated LLPS model accounting for the underlying properties of the chromatin matrix may be required to account for these observations [84].

Internal contacts within TADs occur 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% [64]. 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

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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: Reinterpreting the Link Between Chromatin Folding and Gene Expression

An early study reported that single-cell RNA expression and TAD compaction were correlated [35]. Interestingly, volumes of TADs within inactive and active X-chromosomes were anticorrelated. 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 [64,66]. 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 spatiotemporal 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 rehybridization. We observed that TADs emerge primarily between nuclear cycles 13−14, consistent with previous findings by Hi-C [29]. Strikingly, chromatin within the *snail* TAD was locally decondensed in nuclei displaying *snail* expression. This local decondensation led to an internal reconfiguration of the TAD structure, consistent with genome architecture being affected by, or being prepared for, transcriptional activation during embryo development [64]. These results are consistent with local Polycomb-mediated decondensation occurring before transcriptional activation [85].

Mateo et al. developed a conceptually similar methodology to simultaneously visualize RNA and DNA in single cells [66]. In this approach, cryosections of *Drosophila* embryos were first hybridized with RNA probes and sequentially imaged to obtain the localization patterns of 30 RNA species. Next, the same cryosection was rehybridized 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 [86]. Overall, these studies are consistent with E-P contacts being required to initiate transcription but not for elongation. Many genes are transcribed by episodic bursts of RNA synthesis [87−89]. 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 [90]. 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* [91]. 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 genomewide 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 [92,93]. For

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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 [86]. The extension of these technologies to enable live, multiplexed detection of more species would uniquely permit the dissection of how chromatin is pre-/reorganized 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 [64,66]. 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 [40,42,43,57]. 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 [94] or electron microscopy [95]; 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.

## Conflicts of interest

The authors declare no competing interests.

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