

Redefining enhancer action: insights from structural, genomic, and single-molecule perspectives

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Abstract

This review explores recent emerging insights into enhancer action, focusing on underexplored aspects such as the physical size of regulatory elements, the stochasticity of transcription factor binding and chromatin structure, and the role of nonlinear processes in reconciling longstanding discrepancies between theoretical models and experimental observations. Together, these insights provide a nuanced view of enhancer biology, highlighting the complexity of gene regulation and the need for innovative methodologies to further decode enhancer mechanisms.

Introduction

Enhancers are genomic sequences capable of regulating transcription in an orientation-independent manner [1], and their main role is to tune cell-type specific gene expression patterns in space and time during development and in response to external cellular inputs. Enhancers contain the binding sites of multiple transcription factors (TFs) to recruit different co-activators and co-repressors [2]. Thus, enhancer action can be flexibly modulated amongst cell-types by tuning the composition of TFs a cell expresses. A surprising activity of enhancers is their ability to act over large genomic distances (tens to thousands of kbp) to selectively regulate their target gene, often bypassing multiple other genes whose expression they do not influence [3]. This exquisite specificity imposes clear constraints on models of enhancer action.

Several mechanisms have been proposed to explain how enhancers communicate with their target promoters to regulate gene expression effectively, with three gaining particular interest: stable looping [2], hit-and-run [2,4], and phase-separated condensates [5,6]. Each model, supported by varying degrees of experimental evidence, provides unique insights into

enhancer-promoter mechanisms: stable looping and hit-and-run models focus on chromatin proximity and dynamics, while condensate models highlight biophysical properties and spatial nuclear organization.

Several recent reviews have addressed how technical advances are reshaping our understanding of enhancer regulation [2,7], revisited the debate over whether physical proximity is required for transcriptional activation [8], or explored how chromatin architecture influences transcriptional bursting and enhancer-promoter dynamics [9]. Importantly, gene regulation often extends beyond local enhancer-promoter interactions and involves mechanisms enabling long-range communication within and between TADs. Recent studies have highlighted the role of *facilitator elements*—weak or non canonical enhancers that act in concert with stronger enhancers to support transcriptional regulation across medium (50–400 kb) and long genomic distances (>400 kb) [10–12]. The positional specificity of enhancer activity within regulatory domains, as recently demonstrated at the Sox2 locus [13], adds another layer of complexity that we will not discuss in detail here. In this review, we will instead focus on recent developments in areas that have not been covered in depth elsewhere: (1) the physical dimensions of enhancers, promoters, and their associated molecular players; (2) the dynamics and heterogeneity of transcription factor binding and chromatin structure; (3) how instrumental limitations shape our interpretation of enhancer function; and (4) the role of nonlinear effects on enhancer action.

Spatial and genomic scales in perspective

A major challenge in understanding enhancer function lies in deciphering how they achieve their regulatory roles across diverse spatial and temporal scales. This includes the coordination of gene expression within the complex chromatin and nuclear environment, where enhancer activity must overcome molecular noise, crowding and heterogeneity to produce robust and context-specific outcomes. However, before diving into the complexity of enhancer actions and the potential theoretical models associated with them, it is crucial to first bear in mind the spatial scales and structural constraints inherent to both chromatin and enhancers.

Obtaining approximate, but realistic estimates for the spatial dimensions and the structural organization of the molecular complexes assembled at enhancers and promoters is critical to evaluate enhancer models. Indeed, TF sizes typically range between 4–6 nm [14,15] (Fig. 1a). Once bound, TFs recruit additional cofactors, such as co-activators (p300/CBP), chromatin remodelers (e.g. MLL or BAF complexes), or the mediator complex, to remodel chromatin, reshape histone modifications, and recruit and activate the basal transcriptional machinery [14,16]. These multi-subunit complexes, such p300/CBP, BAF, or MLL can reach sizes between ~10 and ~25 nm (Fig. 1a) [17–19]. Other complexes are even larger, with the mediator and the RNA polymerase II (Pol II) pre-initiation complex (PIC) reaching approximately 30 nm [20,21] and the open cohesin complex exceeding ~40 nm in length [22].

On the other hand, it is important to notice that physical and genomic distances are connected through a power-law, therefore two unlooped genomic loci (e.g. an enhancer and a promoter)

located at relatively short genomic distances (e.g. 5 kb) can be found in average at relative large physical distances (\sim 120 nm) in mice tissues [23] (Fig. 1b, left panel). In contrast, an unlooped EP pair located at relatively large genomic distances (e.g. 250 kb) is not so far away as one may naively expect (Fig. 1b, right panel). Thus, thanks to this nonlinear relation and the high dynamics of chromatin, enhancers and promoters can frequently come into molecular proximity despite their large genomic separation (see next section). Additionally, it is worth noting that enhancers with large genomic footprints (\sim 10 kb for super-enhancers) can themselves occupy large physical volumes (\sim 100-150 nm) (Fig. 1b, middle panel).

We used these estimated sizes to build an approximate structural model of an enhancer getting in molecular contact with a promoter, where the relative sizes of DNA, nucleosomes, TFs and the other complexes involved were at the same scale (Fig. 1c). In contrast to conventional representations, the physical sizes of the complexes involved suggest that a realistic physical distance between enhancers and promoters is approximately 50 nm or higher. We note that at these apparent large distances, enhancer- and promoter-bound complexes would be molecularly contacting each other. As we will argue in Section 3, experimental uncertainties currently limit our ability to detect these EP distances precisely enough to be able to confirm or refute these predicted sizes for an enhancer-promoter loop. An intrinsic limitation of structural representations lies in their inability to effectively capture dynamics and stochasticity, both of which are crucial for realistically testing enhancer models—topics that will be explored in the following section.

Dynamics and stochasticity are widespread

Dynamics and stochasticity characterize most processes involved in transcriptional regulation, from the well documented stochasticity in transcription, to the transient and probabilistic nature of transcription factor binding at regulatory elements [24,25]. Recent advances in single-cell imaging and sequencing are starting to show that other aspects of transcriptional regulation are also highly dynamic and heterogeneous, including chromatin accessibility, histone and DNA modifications, 3D chromatin interactions between regulatory and architectural elements, and higher levels of chromatin organization such as topological associating domains (TADs).

Pioneering single-particle tracking methods have established over the last decade that eukaryotic TFs bind to their target sites in a highly dynamic manner [24,25]. More recently, long-read single-molecule sequencing revealed significant variability in TF binding at enhancers, with some enhancers showing TF occupancy in only \sim 15% of cells, while others displaying binding in up to \sim 90% of cells [26]. Thus, despite the rapid kinetics of TF binding, cognate TF sites are often occupied, likely due to the presence of multiple binding sites that nucleate TFs to the enhancer. Experimental support for this model was recently provided by single-molecule imaging in yeast [27]. Additionally, multi-omic single-molecule approaches showed that the dynamics of DNA methylation and the presence of multiple TF motifs affects the stochasticity of TF binding [28,29]. More recently, a new class of single-molecule footprinting methods has emerged—such as Fiber-seq, SAMOSA, and DiMeLo-seq—which leverage adenine methyltransferases and third-generation sequencing to map TF binding and nucleosome

positioning at near-base resolution [30]. These approaches hold great promise for resolving enhancer occupancy and chromatin structure on individual DNA molecules in a haplotype-resolved manner.

In addition to this stochasticity in TF binding, chromatin structure itself is highly variable [31–33]. This variability can be estimated by measuring the proximity frequency between two genomic regions, defined as the frequency of 3D co-localization for a given cut-off distance [31]; whether these regions are close in space depends of course on the cut-off distance used. Frequencies of EP contacts were reported from single-cell chromatin tracing to be highly variable in *Drosophila*, ranging from 3-30% [34]. EP proximity was also reported to be highly variable in mammalian systems [23,35,36], as well as highly dynamic [37,38].

More recently, it has become clear that even long-range chromatin interactions that were assumed from ensemble sequencing methods (e.g. Hi-C) to be stable are also highly variable between single cells. Two live imaging studies have visualized the 3D positions of distant CTCF sites [39,40]. Despite seemingly contrasting results, both studies are consistent with dynamic CTCF-CTCF loops, see discussion in Ref. [7]. Chromatin tracing studies later provided complementary proof that CTCF loops form infrequently [23,41] and vary considerably between mouse tissues [23]. These results are consistent with the dynamic binding of CTCF [26] and the transient cohesin loop anchoring by CTCF [42].

A recent study investigated 3D proximities between insulators in *Drosophila* [43]. Conventional bioinformatics analysis based on averaging 3D interactions from multiple insulator-bound anchors (pile-up analysis) showed that insulators interact in 3D more often than expected (Fig. 2a). However, use of chromatin tracing revealed that these interactions occur in a very small fraction of cells (~ 12%) (Fig. 2b-c). These seemingly contradictory results can be reconciled by visualizing individual pile-ups, and by performing bootstrapped pile-up analysis, where different combinations and numbers of peaks are averaged to get average pile-up signals (Fig. 2d). Critically, this analysis shows a large heterogeneity between single peaks, and that only when tens of peaks are averaged a statistically-significant difference between observed and expected 3D interactions arises. Thus, these results show that 3D interactions between insulator-bound sites in *Drosophila* are also highly stochastic.

On the other hand, repressive chromatin regions bound by Polycomb (Pc) group proteins were proposed to frequently come into close physical proximity to form rosette-like structures [44], or liquid-liquid phase separated compartments [45,46]. Two recent studies addressed these possibilities using chromatin tracing approaches in *Drosophila* embryos and mESCs [47,48]. In *Drosophila*, genetically-distant Pc domains come into 3D proximity only in a small proportion of cells (<10%) (Fig. 2e) and form clusters harboring multiple Pc domains even less frequently (Fig. 2f). Similarly, most long-range Pc contacts did not assemble in hubs in mESCs [48]. Notably, repressed Pc domains are not uniformly compacted, and can intermix with neighboring chromatin [48], with Pc targets segregating away from their repressed chromatin domain when expressed (Fig. 2g) [47]. Overall, these results indicate that Pc domains are structurally flexible and distant Pc targets do not form phase separated compartments.

This heterogeneity is not limited to activating or repressive loops, but also extends to TADs. Multiple single-cell chromatin tracing experiments performed in mouse, human and flies have now clearly established that TADs emerge from statistical averages of highly heterogeneous structures [32,49–53]. A recent review discusses the statistical view of TADs in detail [7], and we will not go over the same arguments here. If TADs arise from statistical averaging, then it makes little sense to talk about ‘average TAD’ structures derived from an ensemble measurement (e.g Hi-C), or about the position of regulatory elements within TADs. While polymer simulations have been instrumental in aiding interpretation of chromatin tracing results [23,41], generating simulations that faithfully recover the experimental conformational space is a current unfulfilled challenge. In the context of enhancer action, the statistical view of TADs implies that regulatory elements are frequently, dynamically and promiscuously exposed to many chromatin regions in their close genomic proximity —or to distant regions brought in physical proximity— that can potentially influence their state (e.g. promoter activation). Because of this promiscuity in 3D contacts, current enhancer models do not yet explain how enhancers specifically discriminate between cognate and non-cognate targets.

In brief, ensemble measurements such as ChIP-seq, ATAC-seq, or Hi-C, can give the false impression of stability, however, recent single-molecule work has clearly established that TF occupation and 3D conformation at multiple scales are highly dynamic and intrinsically stochastic, imposing clear constraints on enhancer action models, which we will explore in the next sections.

Looping models cannot be ruled out by current measurements of EP proximity

Measuring enhancer-promoter (EP) distances precisely is crucial for distinguishing between looping and condensate models, which predict two different outcomes (Fig. 3a). This requires methods with a “capture radius”—the distance within which interactions can be reliably detected—suited to distinguish between these predictions. The capture radius of Hi-C is ~150–200 nm [2,54], while that of micro-C is considerably smaller (~50 nm) [2]. Thus, the recent finding that EP contacts are more readily detected by micro-C than by Hi-C [55,56] (Fig. 3b) is consistent with EP looping interactions occurring at scales below ~50 nm, as expected for looping models. It is worth noting that these ensemble methods cannot distinguish single-molecule heterogeneity, therefore it is unclear whether the multiple EE, EP, PP peaks observed derive from micro-compartments or from heterogeneous mixes (Fig. 3b).

Imaging provides absolute measurements of proximity frequencies that are not confounded by the capture radius, as they directly measure the full pairwise distance function (PDF) between multiple pairs of genomic loci from single cell data (Fig. 3c). However, instrumental uncertainties, such as chromatic aberrations in multicolor DNA-FISH [31] and drift correction errors in sequential imaging [49,50], currently limit the maximum measurement precision to ~50 nm. These limited precision can considerably affect the measurements of EP distances. For instance, in a stable EP loop model, ground truth EP distances assumed to be at ~27 nm would appear as measured EP distances of ~120 nm [2] (Fig. 3d).

In a hit-and-run looping model, where the enhancer gets transiently in molecular contact with the promoter, one would expect a theoretical PDF with two peaks: one corresponding to the looped conformation, and the second to the unlooped. In contrast, the introduction of experimental uncertainties makes the first peak disappear [2] (Fig. 3e). More drastic perturbations in the shape of the PWD function are to be expected for live imaging measurements of EP distances, where localization error and genomic distance of the labeled region, in addition to chromatic aberrations, lead to considerably less precise measurements. Thus, because of experimental uncertainties, measurements of large distances between enhancers and promoters (>200 nm) [34,36,38,50] do not refute looping models, and are in fact consistent with them [2,7]. In other words, action-at-a-distance models are not necessarily required to explain large EP distances.

EP distances may decrease, remain unchanged, or even increase when comparing transcribing and non-transcribing cells, yet these observations remain consistent with looping models. For instance, EP distances can decrease [57], remain unchanged [34,53] or display subtle changes [36,50] between transcribing and non-transcribing states. Later work showed that whether EP interactions change with transcriptional activation depends on developmental stage [58], while subtle changes in EP interactions can be rationalized in terms of nonlinear models (see next section).

A recent study reported that during hormone stimulation contact frequencies by Hi-C increased while, paradoxically, mean EP distances by DNA-FISH also increased [59]. Similarly, comparison of chromatin structure in transcribing versus non-transcribing pancreatic cells by sequential DNA-FISH showed that EP distances increase in transcribing cells [23]. These seemingly paradoxical results can be reconciled by a hit-and-run looping model where the overall 3D volume occupied by the actively transcribed and spliced gene increases in transcribing cells, leading to larger mean EP distances in the unlooped configuration. Therefore, while it may be counterintuitive for EP distances to remain the same or even increase when comparing non-transcribing and transcribing cells, the current evidence from imaging does not rule out looping models. While phase-separated condensate models could explain these results [60], these models cannot explain how enhancers maintain their exquisite specificity to activate their cognate promoters and avoid off-target activation in the large chromatin region entrapped by the condensate, potentially containing multiple non-cognate promoters (Fig. 3a). In the next section, we will discuss how nonlinear models can also provide alternative explanations to current experimental results without requiring phase-separation.

Nonlinear models

Nonlinearity plays a key role in many biological processes, with several lines of evidence suggesting that nonlinear processes affect enhancer action at multiple levels: TF binding, co-factor recruitment, and physical EP proximity.

Eukaryotic TFs recognize sequences that are too short to define unique genomic positions, therefore the cooperative binding of multiple TFs is needed to increase binding affinity and specificity. In fact, increases in the number of TF sites lead to strongly nonlinear effects on gene

expression [61,62]. This is, in part, the result of cooperative TF binding to DNA by TF oligomerization, by protein-protein interactions between TFs, and by changes in the chromatin substrate itself [63,64]. An alternative model proposes that nonlinear relations between number of TFs and gene expression can also arise through TFs acting on different steps of the transcription cycle [65]. However, until recently, how binding of TFs to an enhancer leads to nonlinear changes in gene expression was still poorly understood, in part due to the lack of methods enabling the measurement of gene expression and the binding of multiple TFs in single cells to establish causative relationships.

A first study recently addressed this limitation by using a single-molecule assay to investigate the roles of TFs in co-factor recruitment. In this elegant work, Ferrie *et al.* combined single-molecule tracking of the potent coactivator p300 with mutation analysis to show that TF-interaction domains are necessary for the binding of p300 to chromatin [66]. These findings are inconsistent with p300 associating to acetylated histones through its catalytic domain or by intrinsically disordered regions. In addition, the authors showed that the action of multiple TF-binding domains are required for p300 to be recruited to regulatory sites. The additive (linear) or synergistic (nonlinear) action of TFs in recruiting p300 was not explicitly explored, but it is reasonable to suggest that coordination in the binding of coactivators to multiple TFs is needed to enhance coactivator specificity [66].

More recently, a second study applied an alternative single-molecule method to investigate cooperativity in TF binding and its consequences for gene expression [67]. This impressive work used single-molecule footprinting on a synthetic construct with a minimal CMV promoter, a Citrine reporter, a proximal enhancer composed of different numbers of TetO binding sites, and a TetR-VP48 fusion to mimic a canonical TF. By varying the number of TF binding sites at the enhancer, the authors showed that expression depends nonlinearly on the number of TF sites, whose occupation varied considerably between single cells. Interestingly, the number of bound TFs depended nonlinearly on the number of TF binding sites, yet individual cells displayed only ~50% of the sites simultaneously bound. Strikingly, a two-state model assuming TF-DNA or nucleosome-DNA cannot account for their binding distributions, which require the inclusion of nucleosome-remodeling activities by the BAF co-activator recruited by TetR-VP48. All in all, a modified Telegraph model with a promoter on-rate proportional to the average number of bound TFs reproduces well their results, and suggest that the nonlinearity in the relation between number of TFs at an enhancer and transcriptional output stems from the action of nucleosome remodeling factors increasing average TF occupancy, and not from synergies arising once TFs are bound. Consistently with this model, intermittent binding of Gal4 to an enhancer with multiple Gal4 binding sites considerably increases transcriptional burst duration [27].

Nonlinearity was also recently proposed and shown to take part in the relation between genomic distance and transcriptional output [7]. On one hand, earlier work proposed that the lack of a strong correlation between EP distance and promoter activity could be explained through a nonlinear model of enhancer-mediated regulation [68]. In fact, various studies since have shown that transcriptional output decays nonlinearly with EP contact frequency [69,70], with multiple EP interactions needed to activate the promoter [69]. More recently, Alamos *et al.* provided

evidence for a similar nonlinear mechanism in *Drosophila* using live imaging of a synthetic reporter system to monitor how a single TF binding site regulates promoter activity for different spatial TF concentrations in the morphogen gradient [71]. By modulation of the binding site affinity, the authors showed that the promoter traverses multiple kinetic transition barriers before the onset of transcription. Interestingly, the strength of the enhancer contributes to its ability to activate a promoter at increased genomic distances [69,70,72]. Surprisingly, the expression-EP distance relation is gene specific, and does not seem to depend on promoter sequence or the presence of repressive marks [70]. Future studies will be required to unveil the nature of the kinetic steps required for a distal enhancer to activate a promoter, as it is so far unclear whether these involve histone modifications or other processes [4].

Conclusions

Understanding the spatial dimensions of chromatin and enhancers, their dynamic occupation by different components of the transcriptional machinery, and the stochasticity and promiscuity of 3D chromatin proximities is essential for contextualizing and evaluating enhancer models. For instance, questions about EP communication remain open, as current measurements of EP proximity, influenced by instrumental limitations, do not rule out looping models. Emerging models incorporating nonlinear processes offer promising alternatives to condensate-based mechanisms, and offer a more comprehensive framework to explain experimental observations. In addition to nonlinearity, the action of distant enhancers may involve non-equilibrium processes that consume energy to stabilize dynamic states, accelerate kinetic transitions, increase robustness to noise, or ensure reaction directionality [73]. In fact, it is reasonable to expect enhancer action to require energy consumption, as it involves chromatin remodeling, deposition of histone marks, recruitment and disassembly of specific complexes, loop extrusion, or phosphorylation [74]. We note that many of the approaches discussed involve fixed samples, which may influence interpretations of spatial or dynamic properties; future work using live-cell or fixation-free methods will be essential to validate and extend current models. In future, the development and application of multi-omic single-molecule techniques, combined with targeted genetic perturbations, will be critical to further dissect single-molecule heterogeneity and rigorously challenge existing models.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

DL: conceptualization, writing - original draft, writing - review and editing, writing - figures. ACG: writing - figures, writing - review and editing. MN: conceptualization, writing - original draft, funding acquisition, writing - review and editing, writing - figures.

Acknowledgments

We acknowledge funding from the Agence National de la Recherche (ANR-23-CE12-0023-01) and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Grant Agreement No 724429). ACG also acknowledges funding from the International Society for Neurochemistry (ISN) - CAEN Category 1B and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, PRESTAMO BID PICT 2020/00090).

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Papers of particular interest, published within the period of the review, have been highlighted as:

* of special interest

** of outstanding interest

Please see the separate file [Highlighted_references.pdf] for highlighted articles and notes.

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Figure legends

Figure 1. Structural models of transcription-related complexes and of an enhancer in molecular contact with a promoter.

- (a) Depiction of various transcription-related proteins and enzymatic complexes at scale. From smallest to largest: b-DNA, general and specific transcription factors (TF) (4-6 nm), p300 and MLL complexes [17,18] (10-12 nm), the BAF and Mediator complexes [19,21] (22-25 nm), RNA Polymerase 2 (RNAP2) pre-initiation complex (PIC) [20] (>27 nm) and the cohesin complex [22] (>40 nm).
- (b) Schematic representations of genomic versus average physical distances for two loci (e.g. enhancer and promoter) in unlooped conformations (left and right panels). The middle panel represents the average distance between the ends of an enhancer with a genomic footprint of ~10 kb (e.g. super-enhancer).
- (c) Schematic representation of an enhancer in molecular contact with a promoter. Enhancers recruit TFs and co-activators, including chromatin remodelers and histone modifiers (p300, MLL, BAF complexes) as well as Mediator. The promoter-bound complex includes general and specific TFs , and the RNAP2 PIC. The Mediator complex

bridges the enhancer-promoter interaction, illustrating the rapid transition to a scale of ~50 nm. Created in <https://BioRender.com>

Figure 2. Single-cell quantification of absolute proximity frequencies between insulators and Pc-bound chromatin are inconsistent with condensate models.

- (a) Aggregate Hi-C plots for non-border regions bond by Class I insulators in nuclear cycle 14 (nc14) *Drosophila* embryos. The colormap indicates $\log_2(\text{Observed}/\text{Expected})$ in a 100 kb window, showing preferential contacts between pairs of insulator-bound loci. Panel adapted from [43].
- (b) Schematic representation depicting Hi-M, an imaging method that allows detection of chromatin tracing and transcriptional status in single cells [49]. Hi-M relies on sequential cycles of probe labeling, imaging and bleaching. Panel adapted from [43].
- (c) Left: schematic representation of the experiment, where multiple insulator-bound and non-insulator chromatin regions are imaged in single cells to detect the frequencies at which they co-localize within a given cut-off distance. Right: Violin plot distributions showing the proximity frequencies between insulator-bound chromatin regions in nc14 embryos. Panel adapted from [43].
- (d) Left: Violin plots illustrating the distribution of mean $\log_2(O/E)$ values for various sets of BEAF-32 anchors across 10,000 iterations. Right: Representative aggregate Hi-C maps for nc14 embryos are displayed for different numbers of BEAF-32 anchors. Panel adapted from [43].
- (e) Upper panel: Schematic of the Hi-M library targeting Pc domains across a segment of approximately 12 Mb on chromosome 3R (*Drosophila*) in wild-type stage 15–16 fly embryos. The three larger domains—ANTP-C, BX-C, and NK-C—are indicated by triangles. Lower left panel: schematic representation of the measurements where the frequency with which each Pc domain interacts with any other Pc domain in chr3R is calculated for a given cut-off radius. Lower right panel: violin plot distributions showing the colocalization frequency to any other Pc domain in chr3R. Adapted from [45].
- (f) Frequency with which different cluster configurations are detected experimentally for different cut-off distances in wild-type stage 15–16 fly embryos. Adapted from [45].
- (g) Top: diagram illustrating segments of stage 15–16 embryos, where Pc genes within the BX-C domain are fully repressed (head) or where at least one target gene is active (A1: Ubx, A7–A9: Abd-A). Middle: simulated and experimental proximity frequency maps for Hox genes within BX-C in the head (H), A1, and A7–9 regions. Bottom: diagrams depicting the segment with the highest proximity between Hox genes (head) and segments where the expression of a gene has lost proximity to other Hox genes within the TAD (bx-Ubx in A1 and Abd-B in A7–9). Adapted from [45].

Figure 3. Experimental uncertainties do not permit ruling out of looping models.

- (a) Structural representations of the looping and condensate models of enhancer action. Top: the looping model predicts interactions at tens of nanometers which should be captured by micro-C, while the condensate model instead suggests at hundreds of nanometers involving non-cognate targets located spread around large genomic regions

(~hundreds of kb). Bottom: corresponding mock contact maps highlighting the expectations from each model.

- (b) Comparison of Hi-C and region capture micro-C (RMCM) contact maps (cut along the diagonal) for a region containing multiple enhancers and promoters. Below the maps, tracks of ChIP-Seq data display structural proteins such as CTCF, epigenetic marks, ATAC-seq, and RNA-seq, offering additional insights into the chromatin landscape of the locus. The contact maps show that EP contacts are more readily detected by RMCM than by Hi-C, consistent with EP contacts often occurring at scales below ~50 nm. Right: schematics representing two models to explain the multiple contacts between enhancers and promoters. On top, a 3D clustering model, below an "ensemble averaging" model where different loops are detected in different cells. Adapted from Ref. [56].
- (c) Left: schematic representation of two labeled regions colocalizing or not colocalizing given a cut-off distance radius. Right: experimental pairwise distance distribution (PWD) of physical distances between two chromatin regions. The blue curve corresponds to a control experiment where the same locus was labeled by two colors, and it is used to define the cut-off distance as the distance at which 99% of the spots are colocalized. The black curve shows the experimental PWD distribution for two genetically distant loci. The proximity frequency is then calculated from the integral of the distribution at a given cut-off radius. Adapted from Ref. [31].
- (d) Left: structural model of the stable loop model. Center: PWD distribution for this model without experimental errors. Right: PWD distribution with experimental uncertainties taken into account (label at 3kb from the enhancer, localization error of 30 and 60 nm in xy, and z, respectively). Due to these uncertainties, a "ground truth" distance of 27 nm would be observed experimentally as a wide distribution with a peak at ~ 120 nm. Adapted from Ref. [2].
- (e) Left: Schematic representation of the hit-and-run looping model. Center: the "ground truth" PWD distribution shows two peaks, corresponding to looped and unlooped conformations. Right: the PDF distribution with experimental uncertainties displays a wide distribution with a single peak, where the first peak appears as a shoulder. Adapted from Ref. [2].

Figure 1

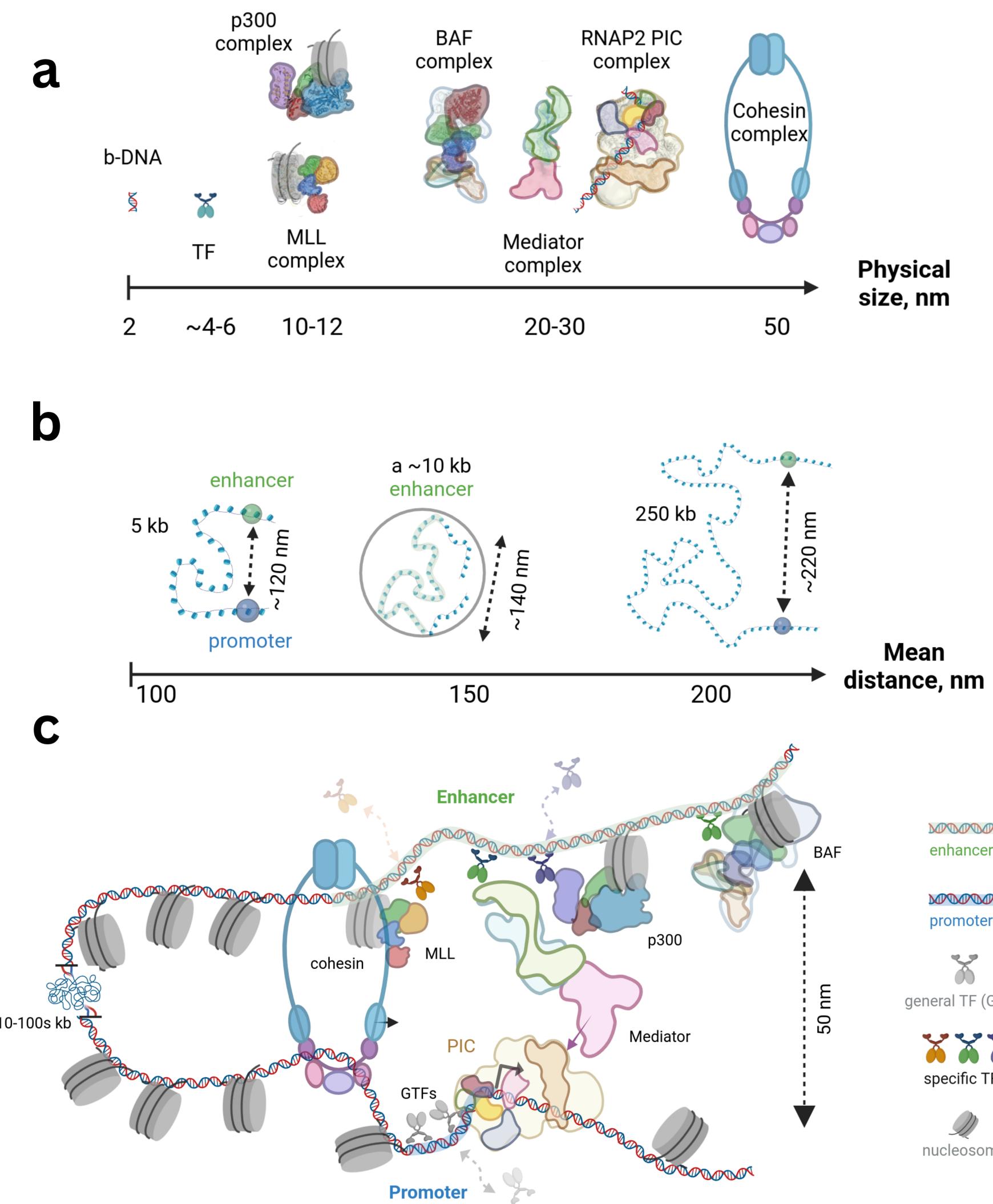


Figure 2

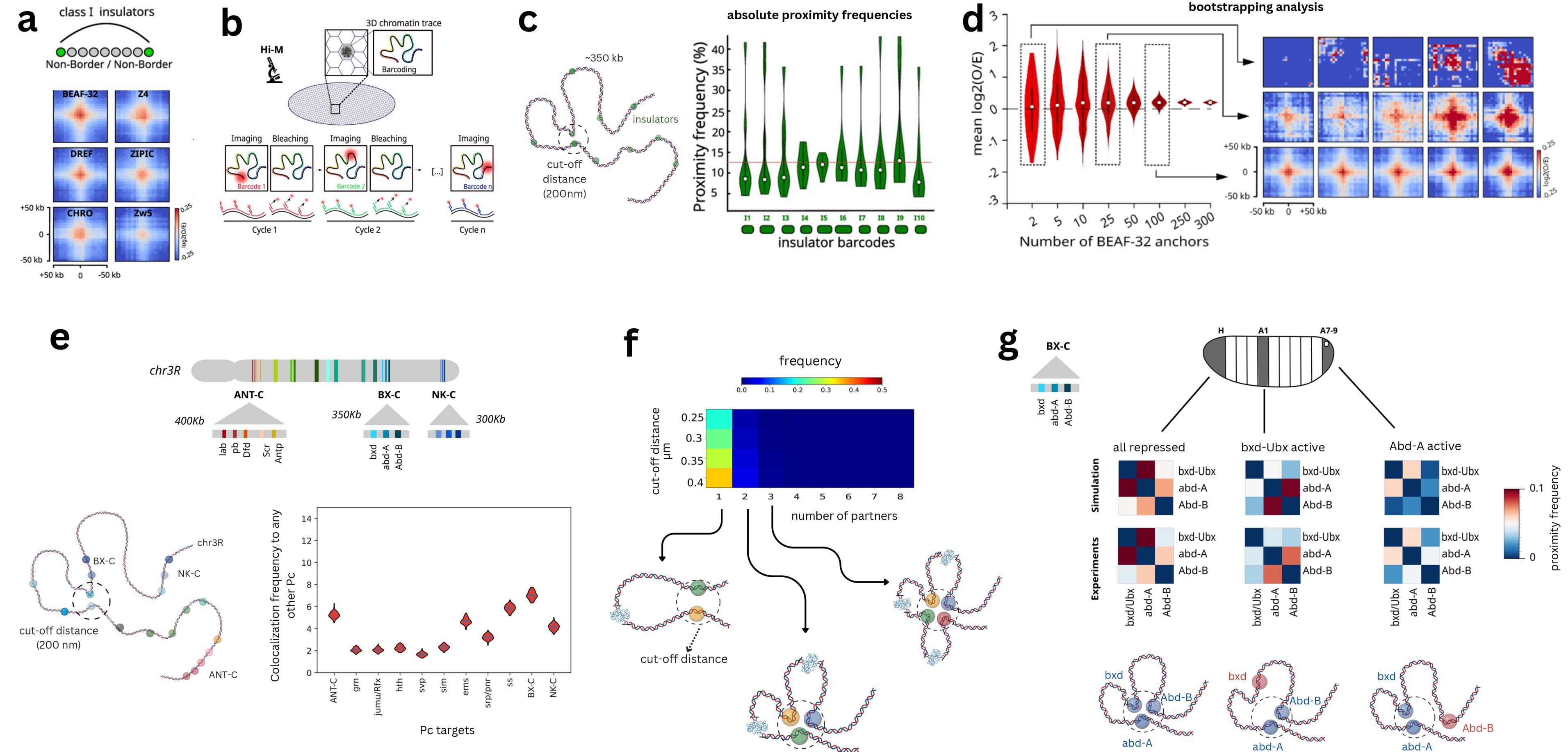
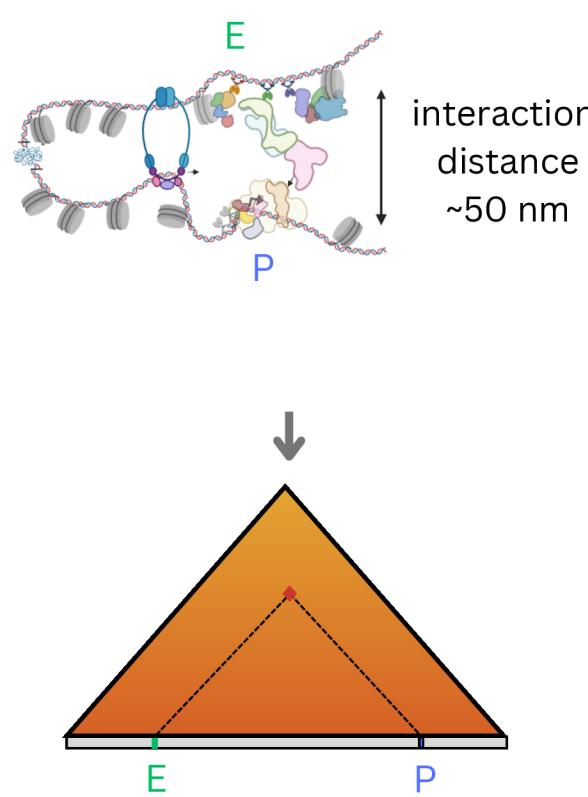
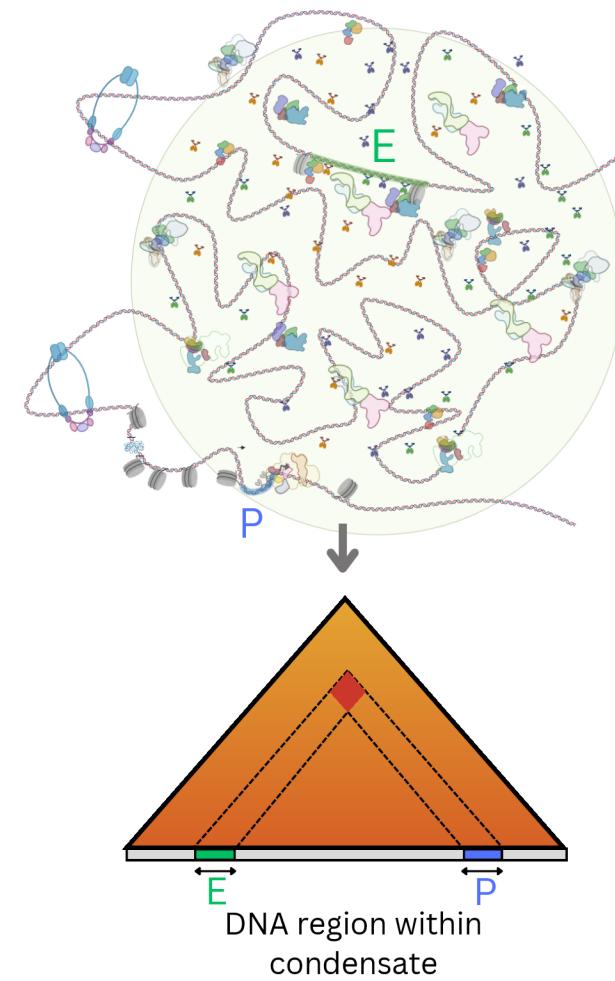


Figure 3

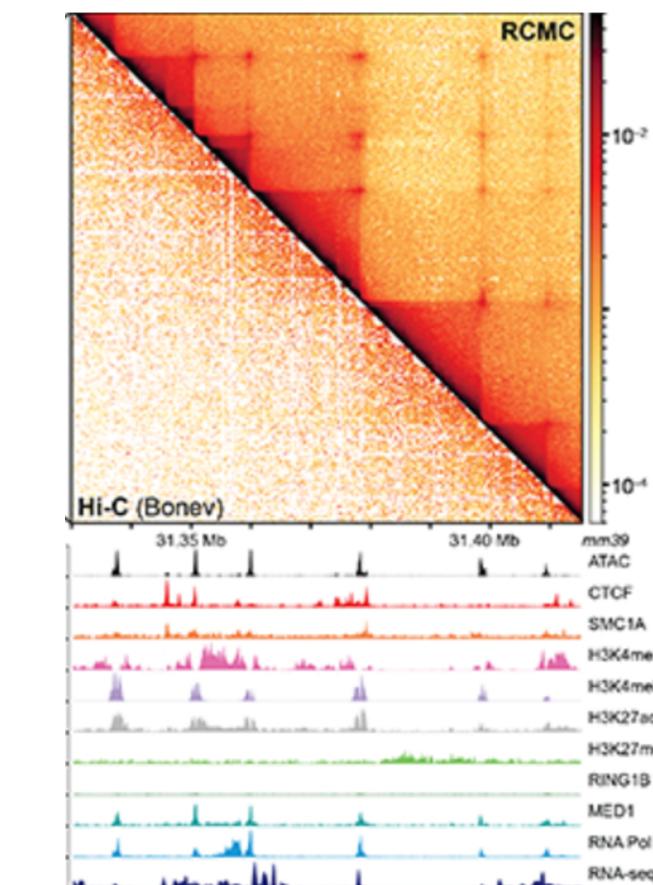
a looping models



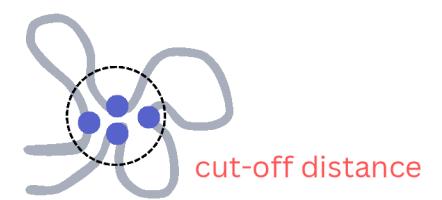
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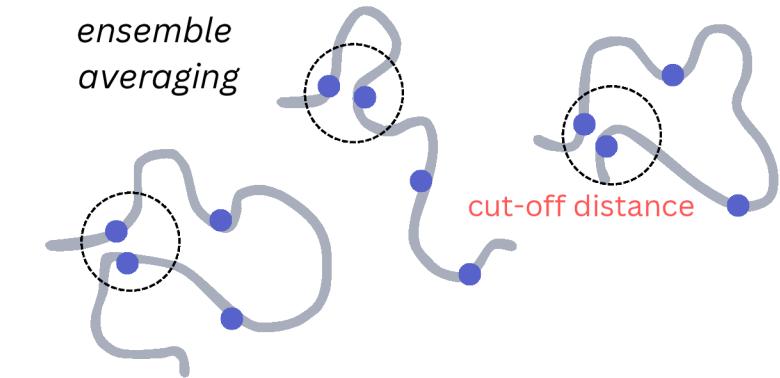
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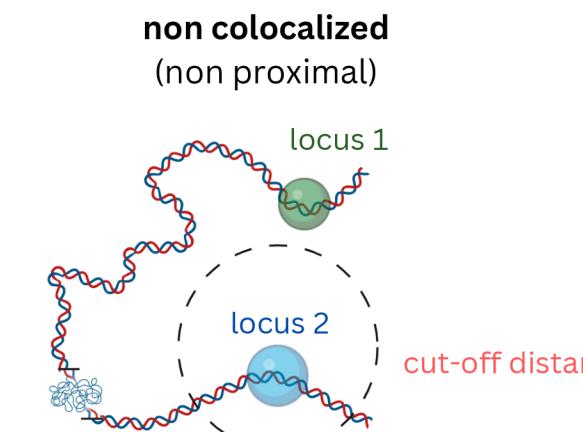
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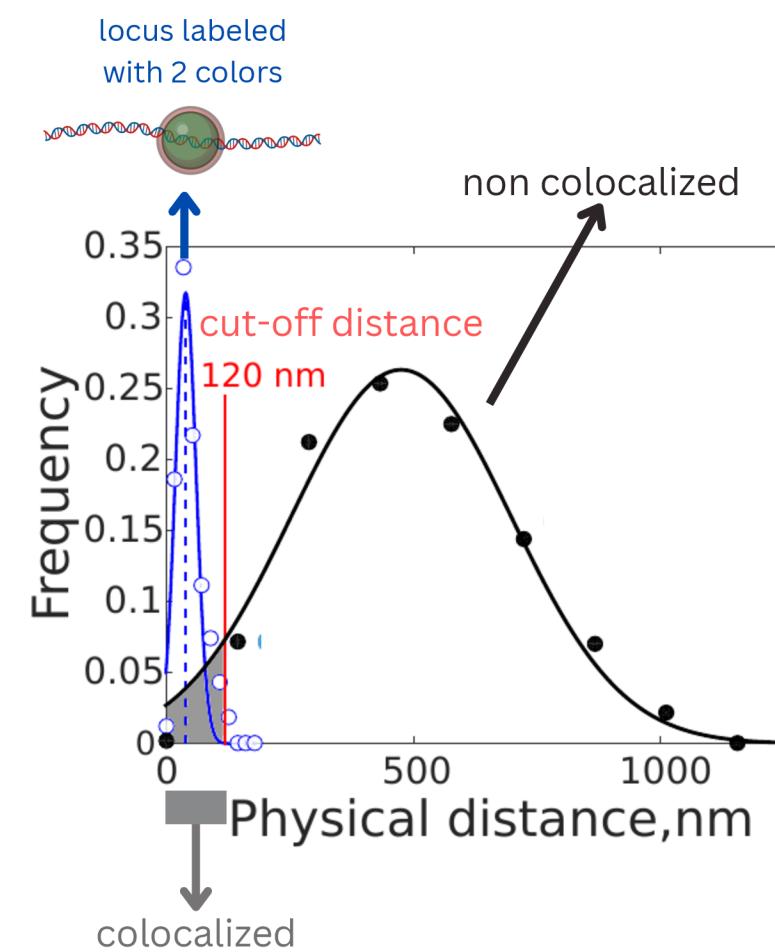
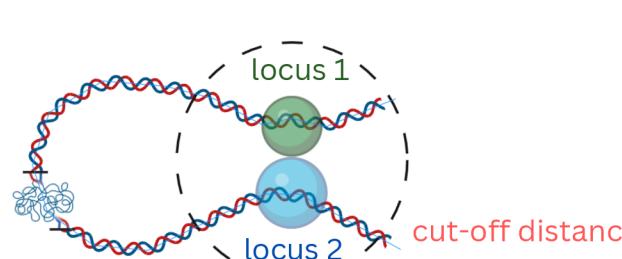
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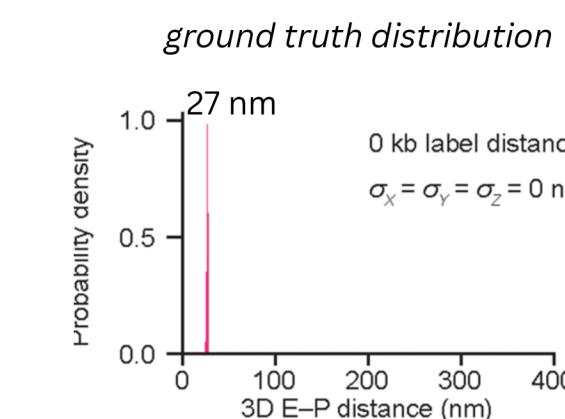
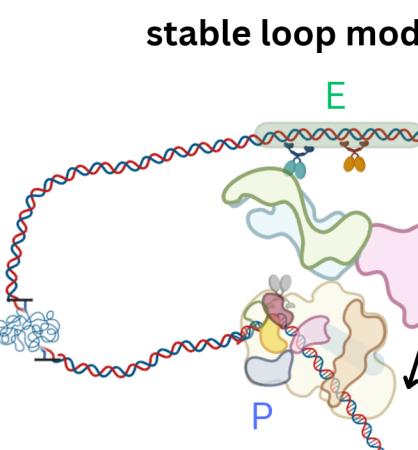
c



colocalized (proximal)



d



e

